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(54) **DNA molecules comprising the genes for preprochymosin and its maturation forms, and microorganisms transformed thereby.**

(57) The present invention relates to recombinant DNA and plasmids comprising specific structural genes of mammalian origin coding for the various allelic and maturation forms of preprochymosin, particularly those of bovine origin, and the use of said recombinant plasmids to transform microorganisms in which said genes are expressed.

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DNA MOLECULES COMPRISING THE VARIOUS ALLELIC FORMS OF THE STRUCTURAL GENES ENCODING THE MAMMALIAN PROTEINS PREPROCHYMOSIN, PROCHYMOSIN, PSEUDOCHYMOSIN AND CHYMOSIN; RECOMBINANT DNA CLONING VEHICLES COMPRISING SAID DNA MOLECULES, MICROORGANISMS TRANSFORMED THEREBY AND SYNTHESIS OF SAID PROTEINS IN THESE MICROBIAL HOSTS

The present invention relates to recombinant DNA and plasmids comprising specific structural genes of mammalian origin coding for the various allelic and maturation forms of preprochymosin, particularly those of bovine origin, and the use of said recombinant plasmids to transform microorganisms in which said genes are expressed.

Chymosin is a protein originating from the stomach of newborn mammals. The bovine type (EC 3.4.23.4) is secreted as an inactive precursor, prochymosin, which consists of a single polypeptide chain of 365 amino acid residues (B. Foltmann *et al.*; Proc. Natl. Acad. Sci. USA; 74, 2321-2324, 1977; B. Foltmann *et al.*; J. Biol. Chemistry; 254, 8447-8456, 1979). Applicants have found that the precursor of bovine prochymosin, preprochymosin, consists of a single peptide chain of 381 amino acid residues and differs from prochymosin in an amino-terminal of 16 amino acids. This extension very much resembles a signal sequence which is involved in the process of cotranslational excretion (G.Blobel & D.Dobberstein, J. Cell. Biol. 67, 835-851, 1975).

Prochymosin is irreversibly converted into active enzyme (chymosin) by limited proteolysis, during which a total of 42 amino acid residues are released from the amino-terminal part of the peptide chain. The activation is effected through a pH-dependent two-step autocatalytic conversion. The intermediate, pseudochymosin, is formed by proteolytic cleavage of bond 27-28 at pH 2-3. The final product, chymosin, is formed by activation of pseudochymosin at pH 4-5. (V. Barkholt Pettersen *et al.*, Eur. J. Biochem., 94, 573-580, 1979).

The enzymatic activity of chymosin consists of the specific proteolysis of κ-casein. This property makes chymosin widely used as milk-clotting enzyme in cheese manufacture.

Chymosin is the essential milk-clotting component of rennet, the crude extract of the abomasum of bovine calves. Rennet is used in the produc-

tion of several types of cheeses in almost all parts of the world. Because of the ever increasing shortage of calf rennet created by the increasing demand for cheese, many laboratories have been searching for substistutes of microbial origin. Many microbial proteases have been screened, only a few could be used in cheese making, and even these substitutes exhibit a different specificity and therefore may cause an unacceptable texture and/or bitter taste of the cheese. So the production of chymosin by recombinant DNA-containing micro-organisms is expected to become of great economical importance.

Developments in recombinant DNA technology have made it possible to isolate or synthesize specific genes or portions thereof from higher organisms, such as man and other mammals, and to transfer these genes or fragments to microorganisms such as bacteria or yeasts. The transferred gene is replicated and propagated as the transformed micro-organism replicates. As a result, the transformed microorganism may become endowed with the capacity to make whatever protein the gene or gene fragment encodes, whether it is an enzyme, a hormone, an antigen, an antibody, or a portion thereof. The microorganism passes on this capability to its progeny, so that in effect, the transfer has resulted in a new microbial strain, having the described capability. See, for example, Ullrich, A. *et al.*, Science 196, 1313 (1977), and Seeburg, P.H. *et al.*, Nature 270, 486 (1977). A basic fact underlying the application of this technology for practical purposes is that DNA of all living organisms, from microbes to man, is chemically similar, being composed of the same four nucleotides. The significant differences lie in the sequences of these nucleotides in the polymeric DNA molecule. The nucleotide sequences are mainly used to specify the amino acid sequences of proteins that comprise the organism. Although most of the proteins of different organisms differ from each other, the coding relationship between nucleotide sequence and amino acid sequence is fundamentally the same for all organisms.

For example, the same nucleotide sequence which codes for the amino acid sequence of HGH in human pituitary cells, will, when transferred to a microorganism, be recognized as coding for the same amino acid sequence.

For economic reasons it is important that proteins encoded by the

recombinant DNA gene are produced under optimal conditions in approved edible micro-organisms as host cells. The main routes to achieve this are:

(1) integration of the structural gene down-stream of an effective regulon, in such a way that under selected growth conditions, the amount of protein produced per cell (by an optimal number of cells) is as high as possible.

For that purpose regulons like the double lac UV5, the trp regulon, the double trp regulon of *E. coli* and the regulon of the gene VIII product of the bacteriophages M13, fd and f1 are, amongst others, adequate in their natural state or in their modified form(s).

Another factor influencing the yield of required protein per cell is the increase (amplification) of the copy number of the plasmids containing the above-described regulons and the structural genes. Amplification can be effected by the use of a thermosensitive replication mutant derived from the cloacin DF 13 plasmid (pVU 208).

(2) excretion of said protein by microbial host cells into their periplasmic space and/or into the culturing medium, thus preventing said protein from intracellular degradation or preventing the disturbance of normal cellular processes due to too high an intracellular level of said protein. It is now generally accepted that in many prokaryotic and eukaryotic cells a special NH_2-terminal amino acid sequence of the unprocessed form of the proteins is involved in the protein excretion process.

G.Blobel & B.Dobberstein (1975), J. Cell Biol. 67, 835-851.

In the present invention use is made of recombinant DNA and other molecular biological techniques to construct recombinant DNA molecules that fulfil the above-described requirements. The present invention is also related to the change of the genetic information of structural genes using site-directed mutagenesis.

For a better understanding of the invention the most important terms used in the description will be defined :

An operon is a gene comprising a particular DNA sequence (structural) gene(s) (for polypeptide(s) expression) and a control region or regulon (regulating said expression) and mostly consisting of a promotor sequence, an operator sequence, a ribosome binding- or interaction DNA

sequence.

Structural genes are DNA sequences which encode through a template (mRNA) a sequence of amino acids characteristic for a specific polypeptide.

A promoter is a DNA sequence within the regulon to which RNA polymerase binds for the initiation of the transcription.

An operator is a DNA sequence within the regulon to which a repressor protein may bind, thus preventing RNA polymerase from binding to the adjacent promoter.

An inducer is a substance which deactivates a repressor protein, freeing the operator and permitting RNA polymerase to bind to the promoter and start transcription.

Cloning vehicle. A non-chromosomal double-stranded DNA, plasmid or phage, comprising a DNA sequence (intact replicon) that allows self-replication after transformation into suitable host cells.

Phage or bacteriophage. Bacterial virus which can replicate in a suitable bacterial host cell.

Reading frame. The grouping of triplets of nucleotides (codons) into such a frame that at mRNA level a proper translation of the codons into the polypeptide takes place.

Transcription. The process of producing RNA from a structural gene.

Translation. The process of producing a polypeptide from mRNA.

Expression. The process undergone by a structural gene to produce a polypeptide. It is a combination of many processes, including at least transcription and translation.

Thermosensitive replication mutant. A plasmid containing a mutation in its replication origin which causes its copy number to be temperature dependent.

Allelic form. One of two or more naturally occurring alternative forms of a gene product.

Maturation form. One of two or more naturally occurring forms of a gene product procured by specific processing, e.g. specific proteolysis.

Plus strand. DNA strand whose nucleotide sequence is identical with the nRNA sequence, with the proviso that uracil is replaced by thymidine.

By maturation forms of preprochymosin are meant prochymosin, pseudochymosin and chymosin.

Prochymosin arises through the action of signal peptidase on preprochymosin, which causes the loss of the amino terminal - excretion related - signal sequence.

The chemical structure of bovine preprochymosin is given in Fig. 1. Bovine prochymosin corresponds with residues 1-365 (Fig. 1).

Pseudochymosin arises through the autocatalytic proteolysis of prochymosin at pH 2, causing the loss of an amino terminal portion of prochymosin. The chemical structure of bovine pseudochymosin corresponds with residues 28-365 of preprochymosin given in Fig. 1.

Chymosin arises through the autocatalytic proteolysis of pseudochymosin at pH 4-5, causing the loss of an amino terminal portion of pseudochymosin. The chemical structure of bovine chymosin corresponds with residues 43-365 of preprochymosin given in Fig. 1.

According to the invention there is provided structural gene(s) coding for the various allelic and maturation forms of mammalian preprochymosin, particularly bovine preprochymosin according to Fig. 1 and 2; and further a recombinant plasmid comprising :

(i) structural gene(s) coding for the various allelic and maturation forms of mammalian preprochymosin, particularly bovine preprochymosin according to Fig. 1 and 2;

(ii) specific DNA sequences which regulate the expression of said structural genes in a microbial host.

These specific DNA sequences consist of either an inducible or a constitutive regulon. A preferred inducible regulon consists of a double lac UV5 system as described by D.V.Goeddel *et al.*, Nature 281, 544-548 (1978). (See Fig. 8).

Another preferred inducible regulon is a constituent of the tryptophan system described by F. Lee *et al.*, J. Mol. Biol. 121, 193-217 (1978) and K. Bertrand *et al.*, Science 189, 22-26 (1975). Applicants have modified this tryptophan system to obtain a more adequate system according to Fig. 9 . In this modified system the information coding for the trp attenuator protein is eliminated while maintaining its ribosome-binding site. Expression is highly increased when two trp regulons in a head to tail fashion are being used. Synthesis of this system is illustrated in Fig. 11.

The recombinant plasmid according to the invention may comprise DNA sequences which regulate the expression of the structural genes, preferably consisting of a modified promoter/ribosome-binding site of gene VIII of bacteriophage M13, fd or f1 (P.M.G.F. van Wezenbeek *et al.*, Gene 11, 129-148 (1980)).

Efficiency of required protein per cell was highly increased when in conjunction with the regulon systems described above, the copy number of the recombinant cloning vehicle was increased. This was effected by the use of a thermosensitive replication mutant derived from the cloacin DF13 plasmid pVU 208 (A. Stuitje, thesis, V.U. Amsterdam, 1981). Increase in temperature results in a ten- to hundred-fold increase in copy number. The construction of such plasmids is illustrated in Fig. 12 and 13.

In the recombinant plasmid according to the invention the regulon may be either directly linked to the structural gene or indirectly through a novel start codon and EcoRI-site containing DNA linker comprising the nucleotide sequence 5'p $CAT(N)_nGAATTC(N')_nATG$ (3') wherein n = 0, 1, 2 or 3 and OH
N and N' are any of the nucleotides A, T, G or C, with the proviso that in the double-stranded structure N and N' are such that a rotational symmetrical structure is present.
By a rotational symmetrical structure is meant that where N is e.g. represented by A, N' should be represented by the complementary base T. In some instances it turned out that the yield of expression improved when the sequence AATT between the regulon and the structural gene has been eliminated.

Allelic forms of bovine preprochymosin have been constructed departing from the nucleotide sequence given in Fig. 1. Examples of these constructions are outlined in Fig. 18 and construction step 10e described further in the Specification, whereby use is made of site-directed mutagenesis. The latter procedure could also be applied usefully so as to produce preprochymosin with a specifically altered signal sequenece which allows efficient excretion in microbial hosts and prochymosin with an improved acid-induced autocatalytic proteolysis characteristic.

The microbial cloning vehicles containing the structural genes encoding the various allelic and maturation forms of preprochymosin according to the invention are produced by a number of steps, the most essential of which are:

1. Isolation and enrichment of the messenger RNA(mRNA) of preprochymosin.
2. Conversion of this mRNA into double stranded DNA (dsDNA)
3. Construction of dsDNA having a poly-dC-tail
4. Incorporation of the dsDNA-poly-dC molecules in PstI-cleaved and poly-dG-tailed pBR 322 DNA.
5. Transformation of competent *E. coli* cells and selection of tetracycline resistant colonies.
6. Determination of the nature of the inserts by RNA/DNA hybridization and *in vitro* translation; and by DNA/DNA hybridization using a specific ^{32}P-labelled cDNA probe.
7. Double checking the nature of the cloned PstI-inserts by DNA- and RNA-sequence analysis.

8a. Producing DNA encoding the amino terminal part of pseudochymosin plus a translational initiation ATG-codon (added) at the amino terminus.

8b. Producing DNA encoding the amino terminal part of chymosin plus a translational initiation ATG-codon at the amino terminus.

8c. Producing DNA encoding the amino terminal part of prochymosin plus a translational initiation ATG-codon at the amino terminus.

8d. Producing DNA encoding the amino terminal part of preprochymosin plus a transitional initiation ATG-codon at the amino terminus.

9. Construction of plasmids comprising a constitutive or inducible regulon, with or without a thermosensitive replication mutation.
10. Construction of plasmids consisting of plasmids described under 9 and the ligated preprochymosin gene or its various maturation

forms, and transformation of said plasmids into microbial host cells.

11. Culturing of microbial cells, e.g. *E. coli* cells, containing recombinant plasmids described under 10 and detection and isolation of preprochymosin, prochymosin, pseudochymosin or chymosin therefrom. Culturing conditions were optimized as to the yield of preprochymosin or its maturation forms per cell. Conversion into enzymatically active chymosin of the various precursors was also optimized.

The following description will illustrate the above-mentioned steps in detail.

1. Isolation and purification of bovine (preprochymosin) mRNA

The fourth stomach of a preruminant calf (abomasum, Frisian cow) was ground under liquid nitrogen, extracted with phenol and a selective precipitation of the RNAs with LiCl was performed following the procedure described by K.S. Kirby, Biochem. J. 96, 266-269 (1965), U. Wiegers & H. Hilz (FEBS. Letters, 23, 77-82 (1972). PolyA-containing mRNA was recovered by several passages over oligo-dT-cellulose columns as described by H. Aviv & P. Leder (Proc. Natl. Acad. Sci. USA, 69, 1408-1412 (1972)).

2. Conversion of (prepro)chymosin mRNA into double-stranded DNA

Purified (prepro)chymosin mRNA was copied with AMV reverse transcriptase to yield a single-stranded DNA molecule, according to the procedure described by G.N. Buell *et al.*, J. Biol. Chem. 253, 2471-2482 (1978). This cDNA was subsequently converted into a double-stranded molecule using DNA-polymerase, according to the procedure described by A.R. Davis *et al.*, Gene 10, 205-218 (1980). Thereafter the loop structure of the double-stranded DNA copy was removed by S1-nuclease digestion.

3. Construction of double-stranded DNA with poly-dC-tails

DNA molecules of the desired length were obtained by polyacrylamide gel-electrophoresis, extracted from the gel and tailed with poly-dC by terminal transferase according to the procedure described by R. Roychoudhury *et al.*, Nucleic Acids Research 3, 863-877 (1976).

4. Integration of the dsDNA-poly-dC molecules in plasmid pBR 322

Plasmid pBR 322 was treated with restriction endonuclease PstI, that cleaves the plasmid at a recognition site that lies in the gene encoding the ampicillin resistance, whereafter the linearized DNA of pBR 322 was supplied at the PstI-site with poly-dG-tails by terminal transferase. The poly-dC-tailed DNA molecules were annealed to the poly-dG-tailed plasmid pBR 322.

5. Transformation and clone selection

The plasmids thus obtained were transferred into $CaCl_2$-treated *E. coli* cells. After transformation, cells containing hybrid plasmid DNA molecules were selected on their resistance to tetracycline. (M. Mandel & A. Higa, J. Mol. Biol., 53, 159-162 (1970).

6. Determination of the nature of the inserts (I) DNA/DNA colony hybridization and RNA/DNA hybridization/*in vitro* translation

Positive colonies were further selected by colony hybridization (R.E. Thayer, Anal. Biochem., 98, 60-63 (1979)) with a radioactively labelled, chymosin-specific cDNA. The latter product was obtained by priming reverse transcription (see 2) of the mRNA preparation (see 1) with the oligonucleotide (5') $dTTCATCATGTT_{OH}$ (3'). This oligonucleotide was designed by the Applicants, because it represents one of the two theoretically possible nucleotide stretches coding for the unique amino acid sequence -asN-met-met-asN-, which occurs in the (prepro)chymosin molecule at position 183-186 (Fig.1). Upon priming cDNA synthesis with this undecanucleotide a distinct cDNA product with a chain length of circa 650 nucleotides was obtained. This product was isolated and used as a probe in the colony hybridization experiments. Several positive colonies could be identified and, as a double check on the identity of the cloned DNA, the plasmid DNA from some of these colonies was isolated and used in the hybridization/*in vitro* translation procedure described by J.G. Williams *et al.* (Cell, 17, 903-913 (1979)).

7. Determination of the nature of the inserts (II) by DNA/RNA sequence analysis (Fig. 1)

The nucleotide sequence analysis of the (prepro)chymosin inserts was performed by the chemical degradation procedure as outlined by A.M. Maxam & W. Gilbert in Methods in Enzymology, L. Grossman &

K. Moldave editors, New York, Acad. Press, 1980, Vol. 65 (1), pages 499-560, and the dideoxy/nick translation procedure as outlined by J. Maat & A.J.H. Smith, Nucleic Acid Research, 5, 4537-4545 (1978). Further information on the nucleotide sequence of the (prepro)chymosin mRNA was derived indirectly by primed synthesis by AMV-reverse transcriptase on the (prepro)chymosin mRNA template in the presence of chain-terminating inhibitors, as outlined by D. Zimmern & P. Kaesberg, Proc. Natl. Acad. Sci. U.S.A. 75, 4257-4261 (1978). This screening yielded *inter alia* plasmid pUR 1001 containing an almost complete copy of preprochymosin mRNA.

8a. Production of DNA encoding the amino terminal part of pseudochymosin ATG (Fig. 3, 4)

Numbers refer to the preprochymosin mRNA sequence in Fig. 1, unless indicated otherwise.

Plasmid pBR 322 was cleaved with the restriction enzyme HaeIII and the resulting fragments were subsequently blunt-end ligated with synthetic HindIII-linkers (5') dCCAAGCTTGG (3'). The mixture was subsequently incubated with HindIII and phosphatase. The reactions were terminated by protein extraction with phenol/chloroform (50/50 v/v) and the DNA was then cleaved with PstI. The resulting mixture was subjected to polyacrylamide gel electrophoresis and a 148 bp fragment (fragment A, Fig. 3) extending from position 3608-3756 in the pBR 322 DNA sequence (J.G. Sutcliffe, Cold Spring Harbor Symposia on Quantitative Biology, 43, 77-90 (1978)) was isolated from the gel by electroelution.

Plasmid pUR 1001 was cleaved with EcoRI and treated with calf phosphatase. The mixture was extracted with phenol/chloroform and subsequently PstI was added. Resulting fragments were separated by agarose electrophoresis. Fragment B (see Fig. 3) extending from the EcoRI-site at position 549 to the PstI-site in the noncoding sequence of the preprochymosin gene at the carboxy terminal end of the pUR 1001 clone, was isolated.

Fragments A and B were ligated with the large EcoRI-HindIII fragment of pUR 201, 301, 401, 303, 210, 310, 410, 311 (combined called fragment C) yielding pUR 1520, 1530, 1540, 1730, 1820, 1830, 1840, 1930, respectively.

Subsequently plasmid pUR 1001 DNA was cleaved with PstI and resulting DNA fragments were ligated to the synthetic pentanucleotide (5') d_{HO}CTGCA (3'). Following ligation, the mixture was incubated with *E. coli* DNA polymerase, large fragment, in the presence of dGTP in order to make blunt ends. The DNA was phosphorylated using T_4 kinase and ATP, and subsequently supplied with synthetic EcoRI-linkers of the structure (5')dCAT$(N)_n$GAATTC$(N')_n$ATG (3'), wherein n = 0, 1, 2 and 3 and N and N' are any of the deoxynucleotides A, C, G or T, with the proviso that in the double-stranded structure N and N' are such that a rotational symmetric structure is present. The DNA was then treated with EcoRI and resulted in fragment (I) of circa 400 bp in length, which was isolated (Fig. 4).

8b. Production of DNA encoding the amino terminal part of chymosin (Fig. 5)

Plasmid pUR 1001 was cleaved with PstI and the 1300 bp PstI insert was isolated. This DNA fragment was heat-denaturated to produce single strands. The synthetic primer (5') dGGGGAGGTGG (3') was used to produce complementary DNA synthesis starting from position 198 in the direction of the carboxy terminus by the action of EcoRI DNA polymerase, large fragment. Subsequently the DNA was treated with nuclease S1 to procure blunt-ended dsDNA. To this dsDNA the synthetic EcoRI-linker (5') dCAT$(N)_n$GAATTC$(N')_n$ATG (3') was ligated. After digestion with EcoRI and treatment with phosphatase, the DNA was cleaved once more by BglII. The resulting fragment II (Fig. 5) was isolated.

8c. Production of DNA encoding the amino terminal part of prochymosin (Fig. 6)

Plasmid pUR 1001 was treated with HphI, followed by nuclease S1. A 202 base pair long fragment III was isolated (Fig. 6). Fragment III was then ligated to the synthetic EcoRI-linker (5') dCAT$(N)_n$GAATTC$(N')_n$ATG, cleaved with EcoRI and dephosphorylated with phosphatase. The resulting DNA was digested once more by BglII and the resulting fragment IV was isolated.

8d. Production of DNA encoding the amino terminal part of preprochymosin (Fig. 7)

Plasmid pUR 1001 was cleaved with PstI and EcoRI. A fragment of

396 bp was isolated. This fragment was then treated with exonuclease III to procure single-stranded non-complementary DNA (A.J.H. Smith (1979), Nucleic Acids Res. 6, 831-841).

This DNA was hybridized to preprochymosin mRNA under conditions described by G. Akusjärvi and U. Petterson (1978), Proc. Natl. Acad. Sci. U.S.A., 75, 5822-5826. The cDNA synthesis was performed as described under 2. Following heat denaturation, dsDNA was made using DNA polymerase, large fragment, with (5') dAGGTGTCTCG$_{OH}$ (3') acting as a primer. The dsDNA was treated with nuclease S1 and ligated to the synthetic EcoRI-linker dCAT(N)$_n$GAATTC(N')$_n$ATG. After EcoRI cleavage and dephosphorylation with (calf intestinal) phosphatase, the DNA was split once more with BglII. The resulting circa 230 bp long fragment (V) was isolated.

9. Construction of plasmids comprising a constitutive or inducible regulon, with or without a thermosensitive replication mutation

9a. Construction of a plasmid pUR 201 (Fig. 8)

A fragment containing 285 base pairs comprising double lac regulon (lac UV5) was obtained by restriction endonuclease EcoRI cleavage of pKB 268, described by K. Backman & M. Ptashne, Cell 13, 65-71 (1978). This fragment was ligated in the EcoRI-site of pBR 322 DNA. Plasmid DNA with the lac regulon in the right orientation, pUR 200, (Fig. 8) was partly cleaved by EcoRI in the presence of *E. coli* RNA polymerase. The EcoRI cleavage site most distant from the restriction endonuclease HindIII cleavage site was preferentially attacked. The linearized DNA was treated with S1 nuclease, purified by agarose gel electrophoresis, circularized by ligation with T4 DNA-ligase and subsequently transformed into *E. coli*. From the tetracycline-resistant transformants pUR 201 with the correct structure (Fig. 8) was obtained.

9b. Construction of plasmid pUR 301 (Fig. 9)

A DNA fragment of about 510 base pairs containing the trp regulon was obtained by restriction endonuclease HinfI cleavage of ptrp ED5, as described by R.A. Hallewell & S. Emtage, Gene 9, 27-47 (1980). This fragment was cleaved with restriction endonuclease TaqI in the presence of *E. coli* RNA polymerase. The TaqI-site in the trp regulon (described by K. Bertrand *et al.*, Science 189, 22-26 (1975) and F. Lee

et al., J. Mol. Biol. 121, 193-217 (1978)) was selectively protected, thus yielding a fragment containing 234 base pairs comprising the trp regulon (Fig. 9). This fragment was then treated with S1 nuclease, blunt-end ligated with the EcoRI-linker (5') dGGAATTCC$_{OH}$ (3'), cut with EcoRI and subsequently cloned in the EcoRI-site of pBR 322.

Plasmid pUR 300 with the trp regulon in the correct orientation (Fig. 9) was isolated. The EcoRI-cleavage site most distant from the HindIII-site was removed by partial cleavage of pUR 300 DNA by EcoRI in the presence of ethidium bromide and S1 nuclease treatment. Linear DNA molecules were recircularized by T4 DNA ligase. From the tetracycline-resistant transformants pUR 301 with the structure as outlined in Fig. 9 was obtained.

9c. Construction of plasmid pUR 401 (Fig. 10)

A 269 base pairs fragment comprising the gene VIII-promotor was obtained by digestion of RF M13 DNA (DNA sequence 1128-1379 see P.M.G.F. van Wezenbeek *et al.*, Gene 11, 129-148 (1980), with the restriction nucleases TaqI and HaeIII; the TaqI-site was made blunt-ended by a repair reaction with *E. coli* DNA polymerase; the fragment was subsequently partly digested with restriction enzyme MnII. The partial products were treated with T4 DNA polymerase and S1 nuclease and subsequently blunt-end ligated with the EcoRI-linker (5') dGGAATTCC$_{OH}$ (3'), then treated with EcoRI and ligated in the EcoRI-site of the pBR 322. By restriction enzyme analysis and DNA sequencing, a plasmid was isolated in which the EcoRI-cleavage site was located just beyond the ribosome-binding site of the M13 gene VIII DNA sequence. Applicants have found that the plasmids having the M13 regulon from nucleotide 1128 to nucleotides 1291 to 1297 were appropriate regulons for expression. The EcoRI-cleavage site most distant from the HindIII-site was removed essentially as described for pUR 301. The complete construction of pUR 401 is outlined in Fig. 10.

9d. Construction of plasmid pUR 303 (Fig. 11)

Plasmid pUR 300 (9b, Fig. 9) was digested with EcoRI and the 234 bp fragment comprising the trp regulon was isolated. This fragment was ligated to pUR 301 DNA, which previously had been cleaved with EcoRI and dephosphorylated with phosphatase. The ligation mixture

was used to transform competent *E. coli* cells and from the ampicillin-resistant transformants pUR 302 was obtained. This plasmid comprises two trp regulons with identical transcription polarity (Fig. 11). pUR 302 was partially cleaved with EcoRI, in the presence of ethidium bromide, treated with nuclease S1 to generate blunt-ends and the cleaved plasmid DNA's were religated. The ligation mix was used to transform competent *E. coli* cells and from the ampicillin-resistant transformants pUR 303 was isolated, wherein the EcoRI-site in between the two trp regulons in pUR 302 had been removed.

9e. Construction of pUR 10 (Fig. 12)

Plasmid pBR 322 was cleaved with PstI and PvuII, and subsequently dephosphorylated with phosphatase. The 2817 bp long fragment (D, Fig. 12) was isolated.

Plasmid pBR 322 was also cleaved with MboII, treated with nuclease S1 and phosphatase and then cleaved once more with PstI. The 400 bp long fragment (E, Fig. 12) extending from position 3201-3608 (J.G. Sutcliffe, Cold Spring Harbor Symposia on Quantitative Biology, 43, 77-90 (1978) was isolated. Plasmid pVU 208 (A.R. Stuitje, thesis, V.U. Amsterdam (1981)) was cleaved with BamHI and treated with nuclease S1. The 760 bp fragment (F, Fig. 12) containing the replication origin of clo DF 13 with the cop ts mutation, was isolated.

To construct pUR 10, fragments D and E were ligated first, followed by ligation with fragment F. The ligation-mix was used to transform competent *E. coli* cells. From ampicillin- and tetracycline-resistant transformants pUR 10-containing cells were isolated. The replication-origin-containing fragment is oriented such that the unidirectional replication is in a counter-clockwise direction.

9f. Construction of pUR 210, pUR 310, pUR 311, pUR 410 (Fig. 13)

These plasmids are derived from pUR 201, pUR 301, pUR 303 and pUR 401, respectively, and contain the cop ts replication origin of pUR 10. pUR 10 was digested with PstI and BamHI and the 2841 bp long fragment (G, Fig. 13) was isolated by agarose gel electrophoresis and electro-elution.

Each of the plasmids pUR 201, pUR 301, pUR 303 and pUR 401 was digested with PstI and BamHI and the "regulon"-containing fragments

(collectively called H, Fig. 13) were isolated. Fragment G and each of the fragments H in turn were ligated using T4 DNA ligase and the ligation mixes were used to transform competent *E. coli* cells. From ampicillin- and tetracycline-resistant colonies pUR 210-, pUR 310-, pUR 311- and pUR 410-containing cells were isolated.

10. Construction of plasmids comprising a constitutive or inducible regulon and the ligated preprochymosin gene or its various allelic and maturation forms, the latter being under transcriptional control of said regulons, and transformation of said plasmids into *E. coli*

10a. Construction of expression plasmids giving rise to the synthesis of bovine pseudochymosin (Fig. 14)

Plasmids pUR 1520, 1530, 1540, 1730, 1820, 1830, 1840 and 1930 were cleaved with EcoRI and dephosphorylated. Each preparation in turn was ligated with fragment I (8a, Fig. 4). This ligation mix was used to transform competent *E.coli* RRI and from the ampicillin resistant transformants, cells containing pUR 1521, 1531, 1541, 1731, 1821, 1831, 1841 and 1931 were selected which contained fragment I inserted such that the genetic information coding for pseudochymosin was present as a continuous uninterrupted entity.

10b. Construction of expression plasmids giving rise to the synthesis of chymosin (Fig. 15)

Plasmid pUR 1521 was cleaved with HindIII, dephosphorylated and then cleaved once more with BglII. The resulting ∿ 1300 bp long fragment VI (Fig. 15) was purified. The vector fragments C (8a, Fig. 3), in turn, were ligated with fragments II (8b, Fig. 5) and fragment VI. The ligation mix was used to transform competent *E. coli* cells and from ampicillin-resistant transformants cells containing pUR 1522, 1532, 1542, 1732, 1822, 1832, 1842 and 1932 were selected.

10c. Construction of expression plasmids giving rise to the synthesis of prochymosin (Fig. 16)

Vector fragments C (8a, Fig. 3), fragment IV (8c, Fig. 6) and fragment VI (9b, Fig. 15) were ligated and the resulting ligation mix was used to transform competent *E. coli* cells.

From the ampicillin-resistant transformant cells containing pUR

1523, 1533, 1543, 1733, 1823, 1833, 1843 and 1933 were selected.

10d. Construction of expression plasmids giving rise to the expression of preprochymosin (Fig. 17)

Vector fragments C (8a, Fig. 3), fragment V (8d, Fig. 7) and fragment VI (9b, Fig. 15) were ligated and the resulting ligation mix was used to transform competent *E. coli* cells.

From the ampicillin-resistant transformants cells containing pUR 1524, 1534, 1544, 1734, 1824, 1834, 1844 and 1934 were selected (Fig. 17).

10e. Example demonstrating the use of site-directed mutagenesis to create allelic forms of the bovine preprochymosin or its maturation forms, departing from the chemical structure given in Fig. 1 and thereby converting residues 202 and 286 into aspartic acid residues, such in turn or in combination (Fig.18-22).

Plasmid pUR 1001 was cleaved with PstI and the resulting DNA fragments were ligated to the synthetic pentanucleotide (5') $_{HO}$dCTGCA$_{OH}$ (3'). Following ligation, the mixture was incubated with *E. coli* DNA polymerase, large fragment in the presence of dGTP in order to make blunt-ends and phosphorylated with T4 kinase and ATP. The DNA was subsequently supplied with EcoRI-linker (5') dCATGAATTCATG (3') and then treated with EcoRI. A circa 880 bp long fragment extending from the EcoRI-site at position 549 to the carboxy terminal end of the chymosin encoding DNA was isolated. This fragment was subsequently cloned in the EcoRI-site of RFM13 mp 2. Two clones were isolated, M13 1020 and M13 1021, which were different in the orientation of the EcoRI-insert with respect to each other (cf. Fig. 18). M13 1020 contained the coding strand (plus strand); M12 1021 contained the non-coding strand (minus strand). ss.Phage DNA of M13 1020 was converted into double-stranded DNA using *E. coli* DNA polymerase large fragment, dNTP's and (5') dTGGCCATCCCTGTCC (3') i

(675)

or (5') dAAACTCATCGTACTG (3') ii

(928)

in turn as primers, using procedures described by S. Gillam *et al.* (1979); Nucleic Acids Res., 6, 2973-2985.

The underlined bases represent mismatches in the primer/template hybrid. Following transformation of competent *E. coli* JM101.7118 cells (B. Gronenborn & J. Messing, Nature, 272, 375-377 (1978)), phages were screened for the required conversion into the chymosin encoding sequence by plaque hybridization with the ^{32}p labelled pentadecanucleotides i, ii, as probes and DNA-sequence analysis.

Two phage isolates, M13.1022 and 1023 contained the DNA-sequences:

```
                asp arg asp gly
(5') —— G GAC AGG GAT GGC CA —— (3')      M13.1022
                    675

                glN tyr asp glu phe
(5') —— CAG TAC GAT GAG TTT —— (3')       M13.1023
                    928
```

RF M13.1022 and RF M13.1023 were cleaved with EcoRI, dephosphorylated and then cleaved with PstI. From each preparation an 888 bp long fragment was isolated by agarose gel electrophoresis and electroelution. Apart from the required mutations, this fragment corresponds with fragment B (8a, Fig. 3).

Using procedures identical with those described in 8a-8c, expression plasmids were constructed which gave rise to the synthesis of specifically altered pseudochymosin, chymosin, prochymosin and preprochymosin, respectively.

11. Culturing of *E. coli* cells containing recombinant plasmids described under 10 and detection and isolation of preprochymosin, prochymosin, pseudochymosin or chymosin

E. coli cells containing one of the plasmids
pUR 1521 (ATCC 39120), 1531, 1541, 1731, 1821, 1831, 1841, 1932
pUR 1522, 1532, 1542, 1732, 1822, 1832 (ATCC 39197), 1842, 1932
pUR 1523, 1533 (ATCC 39121), 1543, 1733, 1823, 1833, 1843, 1933
pUR 1524, 1534, 1544, 1734 (ATCC 39198), 1824, 1834, 1844, 1944
with or without the AATT-sequence in the linker between the regulon and the preprochymosin gene or its maturation forms were cultured under optimal conditions for their growth. These culturing conditions vary with the type of plasmid present in the cells, but a suitable antibiotic (ampicillin) was always present to maintain selection pressure.

Under these conditions the cells containing either plasmids pJR 1521, 1531, 1541, 1731, 1821, 1831, 1841, 1931 or pJR 1522, 1532, 1542, 1732, 1822, 1832, 1842, 1932 or pJR 1523, 1533, 1543, 1733, 1823, 1833, 1843, 1933 or pJR 1524, 1534, 1544, 1734, 1824, 1834, 1844, 1944 produced considerable amounts of pseudochymosin, chymosin, prochymosin or preprochymosin, respectively. These amounts varied from 10^3-10^7 molecules/cell.

E. coli cells which contained preprochymosin or modified preprochymosin encoding plasmids contained (modified) preprochymosin in the cytoplasm and prochymosin in their periplasmic space.

The bacterially produced preprochymosin, prochymosin and pseudochymosin could be converted into chymosin using the procedures described by V. Barkholt Pedersen *et al.* (Eur. J. Biochem., 94, 573-580 (1979)). The chymosins which were thus obtained and bacterially-produced chymosin were shown to be fully biologically active in proteolysis.

The presence of the proteins was further demonstrated by SDS-polyacrylamide gel electrophoresis with or without immunoprecipitation, and by immunological ELISA and RIA tests. The antisera for this test were generated by injecting bovine calf chymosin supplemented with Freund adjuvant into sheep as well as rabbits.

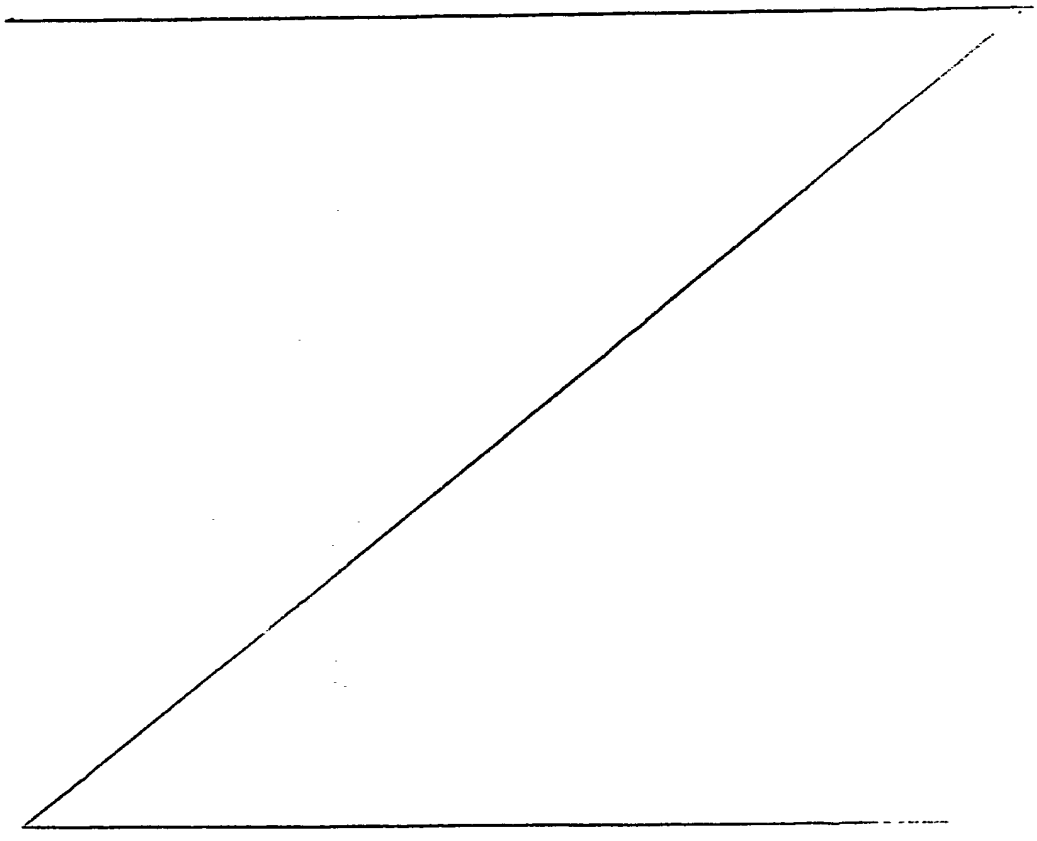
The above description was focussed on the synthesis of chymosin in *E. coli* cells; it is of course possible and desirable to use for that purpose non-toxic, edible micro-organisms, such as streptococci or micro-organisms of Bacillus or yeast origin.

Abbreviations

DNA	deoxyribonucleic acid
cDNA	complementary DNA
dsDNA	double-stranded DNA
RNA	ribonucleic acid
mRNA	messenger RNA
A	adenine
G	guanine
C	cytosine
T	thymine
RBS	ribosome-binding site
N	any nucleotide
bp	pase pair
M13	bacteriophage M13
RF	replicative form
p	plasmid
^{32}P	phosphorus 32
n	number
p/o	promotor/operator
trp	tryptophan
lac	lactose
EcoRI	restriction endonuclease derived from *Escherichia coli* RYI
HindIII	restriction endonuclease derived from *Haemophilus Influenzae* RdIII
HinfI	restriction endonuclease derived from *Haemophilus Influenzae* -
HaeIII	restriction endonuclease derived from *Haemophilus aegypticus*
BglII	restriction endonuclease derived from *Bacillus globii*
HphI	restriction endonuclease derived from *Haemophilus parahaemolyticus*
PstI	restriction endonuclease derived from *Providencia stuartii*
MnlI	restriction endonuclease derived from *Moraxella nonliquefaciens*
TaqI	restriction endonuclease derived from *Thermophilus aquaticus*
BamHI	restriction endonuclease derived from *Bacillus amyloliquefaciens* H
E. coli	*Escherichia coli*
ts	thermosensitive
DdeI	restriction endonuclease derived from *Desulfovibrio desulfuricans*
HpaII	restriction endonuclease derived from *Haemophilus parainfluenzae*
ELISA	enzyme linked immuno sorbent assay
RIA	radioimmune assay
SDS	sodium dodecyl sulphate

Abbreviations (continued)

met	methionine
leu	leucine
ile	isoleucine
ala	alanine
asp	aspartic acid
asN	asparagine
glu	glutamic acid
glN	glutamine
val	valine
thr	threonine
phe	phenylalanine
tyr	tyrosine
cys	cysteine
arg	arginine
ser	serine
his	histidine
pro	proline
gly	glycine
lys	lysine
Ap	ampicillin resistance
Tc	tetracycline resistance



Legends to the Figures:

Fig. 1. Amino acid sequence of one of the allelic forms (B) of bovine preprochymosin and DNA sequence corresponding to the bovine preprochymosin B mRNA.
The S numbering denotes numbering of the signal sequence amino acid residues.

Fig. 2. Schematic representation of several examples of allelic forms of bovine preprochymosin. The upper drawing corresponds with Fig. 1. The numbering in parentheses denotes the amino acid residues in the preprochymosin molecule. Other numbers refer to the nucleotide sequence of the mRNA.

Fig. 3. Construction route of pUR 1520, 1530, 1540, 1730, 1820, 1840 and 1930, described in 8a.

Fig. 4. Construction route of dsDNA encoding amino terminal end of pseudochymosin plus a transcriptional initiation triplet, described in 8a.

Fig. 5. Construction route of dsDNA encoding the amino terminal end of chymosin plus a transcriptional initiation triplet, described in 8b.

Fig. 6. Construction route of dsDNA encoding the amino terminal end of prochymosin plus a transcriptional initiation triplet, described in 8c.

Fig. 7. Construction route of dsDNA encoding the amino terminal end of preprochymosin, described in 8d.

Fig. 8. Construction route of pUR 201, described in 9a.

Fig. 9. Construction route of pUR 301, described in 9b.

Fig. 10. Construction route of pUR 401, described in 9c.

Fig. 11. Construction route of pUR 303, described in 9d.

Fig. 12. Construction route of pUR 10, described in 9e.

Fig. 13. Construction route of pUR 210, 310, 311 and 410, described in 9f.

Fig. 14. Construction route of pUR 1521, 1531, 1541, 1731, 1821, 1831, 1841 and 1931, described in 10a.

Fig. 15 Construction route of pUR 1522, 1532, 1542, 1732, 1822, 1832, 1842 and 1932, described in 10b.

Fig. 16. Schematic representation of pUR 1523, 1533, 1543, 1733, 1823, 1833, 1843 and 1933, described in 10c.

Fig. 17. Schematic representation of pUR 1524, 1534, 1544, 1734, 1824, 1834, 1844 and 1934, described in 10d.

Fig. 18. Construction route of M13.1020, M13.1021 and part of dsDNA encoding an allelic variation of bovine preprochymosin, described in 10e.

Fig. 19. General representation of the plus strand DNA sequence corresponding to the structural gene encoding preprochymosin

wherein :

A is deoxyadenyl,
G is deoxyguanyl,
C is deoxycytosyl,
T is thymidyl,
J is A or G;
K is T or C;
L is A, T, C, or G;
M is A, C or T;
X is T or C if Y is A or G, and C if Y is C or T;
Y is A, G, C or T if X is C, and A or G if X is T;
W is C or A if Z is G or A, and C if Z is C or T;
Z is A, G, C or T if W is C, and A or G if W is A;
QR is TC if S is A, G, C or T, and AG if S is T or C; and
S is A, G, C or T if QR is TC, and T or C if QR is AG.

Fig. 20. General representation of the plus strand DNA sequence corresponding to the structural gene encoding prochymosin plus a transcriptional initiation ATG-triplet wherein :

A is deoxyadenyl,
G is deoxyguanyl,
C is deoxycytosyl,
T is thymidyl,
J is A or G;
K is T or C;
L is A, T, C, or G;
M is A, C or T;
X is T or C if Y is A or G, and C if Y is C or T;
Y is A, G, C or T if X is C, and A or G if X is T;
W is C or A if Z is G or A, and C if Z is C or T;
Z is A, G, C or T if W is C, and A or G if W is A;
QR is TC if S is A, G, C or T, and AG if S is T or C; and
S is A, G, C or T if QR is TC, and T or C if QR is AG.

Fig. 21. General representation of the plus strand DNA sequence corresponding to the structural gene encoding pseudochymosin plus

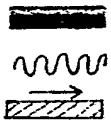
a transcriptional initiation ATG-triplet wherein :
A is deoxyadenyl,
G is deoxyguanyl,
C is deoxycytosyl,
T is thymidyl,
J is A or G;
K is T or C;
L is A, T, C, or G;
M is A, C or T;
X is T or C if Y is A or G, and C if Y is C or T;
Y is A, G, C or T if X is C, and A or G if X is T;
W is C or A if Z is G or A, and C if Z is C or T;
Z is A, G, C or T if W is C, and A or G if W is A;
QR is TC if S is A, G, C or T, and AG if S is T or C; and
S is A, G, C or T if QR is TC, and T or C if QR is AG.

Fig. 22. General representation of the plus strand DNA sequence corresponding to the structural gene encoding chymosin plus a transcriptional initiation ATG-triplet wherein :
A is deoxyadenyl,
G is deoxyguanyl,
C is deoxycytosyl,
T is thymidyl,
J is A or G;
K is T or C;
L is A, T, C, or G;
M is A, C or T;
X is T or C if Y is A or G, and C if Y is C or T;
Y is A, G, C or T if X is C, and A or G if X is T;
W is C or A if Z is G or A, and C if Z is C or T;
Z is A, G, C or T if W is C, and A or G if W is A;
QR is TC if S is A, G, C or T, and AG if S is T or C; and
S is A, G, C or T if QR is TC, and T or C if QR is AG.

General remarks on the figures

Generally plasmid DNA is drawn as a single-lined circle, still this represents double-stranded DNA (bacteriophage M13. DNA is single-stranded; the replicative form RF, however, is double-stranded). 5'-ends of cleaved DNA at restriction enzyme cleavage site are phosphorylated unless indicated otherwise; 3'-ends are always dephosphorylated.

The numbers given in italics refer to the bovine preprochymosin DNA sequence given in Fig. 1; otherwise they refer to plasmid DNA sequences.



dA/dT-stretch

dG/dC-stretch

regulon, the arrow indicates transcription direction

CLAIMS

1. DNA sequences encoding various allelic and maturation forms of mammalian preprochymosin according to Fig. 1 and 2.

2. DNA sequences encoding various allelic and maturation forms of mammalian preprochymosin according to Fig. 19, 20, 21 and 21.

3. A recombinant plasmid comprising

(i) a structural gene coding for the various allelic and maturation forms of mammalian preprochymosin according to Fig. 1 and 2 or 19-22.

(ii) specific DNA sequences regulating the expression of said gene in a microbial host, consisting of an inducible or constitutive regulon.

4. A recombinant plasmid according to Claim 2, wherein the regulon consists of a double lac UV5 system according to Fig. 8.

5. A recombinant plasmid according to Claim 3, comprising a regulon consisting of at least one modified tryptophan system according to Fig. 9, wherein the information coding for the trp attenuator protein is eliminated while its ribosome-binding site is maintained.

6. A recombinant plasmid according to Claim 5, comprising two trp regulons linked in a head to tail fashion according to Fig. 11.

7. A recombinant plasmid according to Claim 3, comprising a regulon consisting of at least one modified promotor/ribosome-binding site of gene VIII of bacteriophage M13, according to Fig. 10.

8. A recombinant plasmid according to Claim 2, comprising a thermosensitive replication mutant derived from the cloacin DF 13 plasmid pVU 208 according to Fig. 12 and 13.

9. A recombinant plasmid according to Claim 1, selected from

pUR 1521 (ATCC 39120), 1531, 1541, 1731, 1821, 1831, 1841, 1931
pUR 1522, 1532, 1542, 1732, 1822, 1832 (ATCC 39197), 1842, 1932
pUR 1523, 1533 (ATCC 39121), 1543, 1733, 1823, 1833, 1843, 1933
pUR 1524, 1534, 1534, 1544, 1734 (ATCC 39198), 1824, 1834, 1844, 1944

10. A bacterial culture comprising E-coli cells comprising a plasmid according to Claim 9.

11. Micro-organisms, particularly non-toxic, edible micro-organisms such as streptococci or lactobacilli, or micro-organisms of bacillus or yeast origin, in which are incorporated a DNA sequence according to Claim 1 or Claim 2, and an appropriate regulon, suitable for the production of bovine calf preprochymosin or any one of the various allelic and maturation forms thereof.

CLAIMS

1. A process for producing DNA sequences encoding various allelic and maturation forms of preprochymosin according to Fig. 1 and 2 or Fig. 19-22, characterized in that :

(a) the message RNA (mRNA) of preprochymosin is isolated and purified;

(b) mRNA is converted into double stranded DNA (dsDNA);

(c) said dsDNA is incorporated in an appropriate plasmid;

(d) said plasmid is transferred into bacterial cells and hybrid plasmid DNA molecules are selected to yield a plasmid containing a substantially complete copy of the preprochymosin mRNA;

(e) this DNA is further genetically engineered so as to code exactly for preprochymosin or its maturation forms and brought under transcriptional control of appropriate regulons.

2. A process for producing a recombinant plasmid, characterized by combining

(i) a structural gene coding for the various allelic and maturation forms of mammalian preprochymosin according to Fig. 1 and 2 or 19-22; with

(ii) specific DNA sequences regulating the expression of said gene in a microbial host, consisting of an inducible or constitutive regulon.

3. A process according to Claim 2, characterized in that the regulon consists of a double lac UV5 system according to Fig. 8.

4. A process according to Claim 2, characterized in that a regulon is used which consists of at least one modified tryptophan system according to Fig. 9, wherein the information coding for the trp attenuator protein is eliminated while its ribosome-binding site is maintained.

5. A process according to Claim 4, characterized in that 2 trp regulons are used which are linked in a head to tail fashion according to Fig. 11.

6. A process according to Claim 2, characterized in that a regulon is used which consists of at least one modified promotor/ribosome-binding site of gene VIII of bacteriophage M13, according to Fig. 10.

7. A process according to Claim 2, characterized in that a thermo-sensitive replication mutant is used which is derived from the cloacin DF13 plasmid pVU 208 according to Fig. 12 and 13.

8. A process according to Claim 2, characterized in that a plasmid is used which is selected from :

pUR 1521 (ATCC 39120), 1531, 1541, 1731, 1821, 1831, 1841, 1931
pUR 1522, 1532, 1542, 1732, 1822, 1832 (ATCC 39197), 1842, 1932
pUR 1523, 1533 (ATCC 39121), 1543, 1733, 1823, 1833, 1843, 1933
pUR 1524, 1534, 1534, 1544, 1734 (ATCC 39198), 1824, 1834, 1844, 1944

9. A process for producing mammalian chymosin, characterized in that

(a) a plasmid according to Claim 2 is incorporated in a micro-organism;

(b) the transformed micro-organism is cultivated and

(c) chymosin produced by said micro-organism is isolated.

10. A process according to Claim 9, characterized in that the micro-organisms consist of E-coli cells.

11. A process according to Claim 9, characterized in that non-toxic edible micro-organisms such as streptococci or lactobacilli, or micro-organisms of bacillus or yeast origin are used.

```
                                    s1                  s5                  s10
                                    met arg cys leu val val leu leu ala val phe ala leu
CAGCG GCT GGA CCC AGA TCC AAG ATG AGG TGT CTC GTG GTG CTA CTT GCT GTC TTC GCT CTC
  3

     s15      1                                                  10
ser gln gly ALA GLU ILE THR ARG ILE PRO LEU TYR LYS GLY LYS SER LEU ARG LYS ALA
TCC CAA GGC GCT GAG ATC ACC AGG ATC CCT CTG TAC AAA GGC AAG TCT CTG AGG AAG GCG
 63

          20                                             *  30
LEU LYS GLU HIS GLY LEU LEU GLU ASP PHE LEU GLN LYS GLN GLN TYR GLY ILE SER SER
CTG AAG GAG CAT GGG CTT CTG GAG GAC TTC CTG CAG AAA CAG CAG TAT GGC ATC AGC AGC
123


          40                                             50
LYS TYR SER GLY PHE GLY GLU VAL ALA SER VAL PRO LEU THR ASN TYR LEU ASP SER GLN
AAG TAC TCC GGC TTC GGG GAG GTG GCC AGC GTG CCC CTG ACC AAC TAC CTG GAT AGT CAG
183

          60                                             70
TYR PHE GLY LYS ILE TYR LEU GLY THR PRO PRO GLN GLU PHE THR VAL LEU PHE ASP THR
TAC TTT GGG AAG ATC TAC CTC GGG ACC CCG CCC CAG GAG TTC ACC GTG CTG TTT GAC ACT
243

          80                                             90
GLY SER SER ASP PHE TRP VAL PRO SER ILE TYR CYS LYS SER ASN ALA CYS LYS ASN HIS
GGC TCC TCT GAC TTC TGG GTA CCC TCT ATC TAC TGC AAG AGC AAT GCC TGC AAA AAC CAC
303

          100                                            110
GLN ARG PHE ASP PRO ARG LYS SER SER THR PHE GLN ASN LEU GLY LYS PRO LEU SER ILE
CAG CGC TTC GAC CCG AGA AAG TCG TCC ACC TTC CAG AAC CTG GGC AAG CCC CTG TCT ATC
363

          120                                            130
HIS TYR GLY THR GLY SER MET GLN GLY ILE LEU GLY TYR ASP THR VAL THR VAL SER ASN
CAC TAC GGG ACA GGC AGC ATG CAG GGC ATC CTG GGC TAT GAC ACC GTC ACT GTC TCC AAC
423
          140                                            150
ILE VAL ASP ILE GLN GLN THR VAL GLY LEU SER THR GLN GLU PRO GLY ASP VAL PHE THR
ATT GTG GAC ATC CAG CAG ACA GTA GGC CTG AGC ACC CAG GAG CCC GGG GAC GTC TTC ACC
483

          160                                            170
TYR ALA GLU PHE ASP GLY ILE LEU GLY MET ALA TYR PRO SER LEU ALA SER GLU TYR SER
TAT GCC GAA TTC GAC GGG ATC CTG GGG ATG GCC TAC CCC TCG CTC GCC TCA GAG TAC TCG
543

          180                                            190
ILE PRO VAL PHE ASP ASN MET MET ASN ARG HIS LEU VAL ALA GLN ASP LEU PHE SER VAL
ATA CCC GTG TTT GAC AAC ATG ATG AAC AGG CAC CTG GTG GCC CAA GAC CTG TTC TCG GTT
603

          200                                            210
TYR MET ASP ARG ASN GLY GLN GLU SER MET LEU THR LEU GLY ALA ILE ASP PRO SER TYR
TAC ATG GAC AGG AAT GGC CAG GAG AGC ATG CTC ACG CTG GGG GCC ATC GAC CCG TCC TAC
663
```

Fig.1

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        220                                             230
TYR THR GLY SER LEU HIS TRP VAL PRO VAL THR VAL GLN GLN TYR TRP GLN PHE THR VAL
TAC ACA GGG TCC CTG CAT TGG GTG CCC GTG ACA GTG CAG CAG TAC TGG CAG TTC ACT GTG
723

        240                                             250
ASP SER VAL THR ILE SER GLY VAL VAL VAL ALA CYS GLU GLY GLY CYS GLN ALA ILE LEU
GAC AGT GTC ACC ATC AGC GGT GTG GTT GTG GCC TGT GAG GGT GGC TGT CAG GCC ATC CTG
783

        260                                             270
ASP THR GLY THR SER LYS LEU VAL GLY PRO SER SER ASP ILE LEU ASN ILE GLN GLN ALA
GAC ACG GGC ACC TCC AAG CTG GTC GGG CCC AGC AGC GAC ATC CTC AAC ATC CAG CAG GCC
843

        280                                             290
ILE GLY ALA THR GLN ASN GLN TYR GLY GLU PHE ASP ILE ASP CYS ASP ASN LEU SER TYR
ATT GGA GCC ACA CAG AAC CAG TAC GGT GAG TTT GAC ATC GAC TGC GAC AAC CTG AGC TAC
903

        300                                             310
MET PRO THR VAL VAL PHE GLU ILE ASN GLY LYS MET TYR PRO LEU THR PRO SER ALA TYR
ATG CCC ACT GTG GTC TTT GAG ATC AAT GGC AAA ATG TAC CCA CTG ACC CCC TCC GCC TAT

        320                                             330
THR SER GLN ASP GLN GLY PHE CYS THR SER GLY PHE GLN SER GLU ASN HIS SER GLN LYS
ACC AGC CAG GAC CAG GGC TTC TGT ACC AGT GGC TTC CAG AGT GAA AAT CAT TCC CAG AAA
1023

        340                                             350
TRP ILE LEU GLY ASP VAL PHE ILE ARG GLU TYR TYR SER VAL PHE ASP ARG ALA ASN ASN
TGG ATC CTG GGG GAT GTT TTC ATC CGA GAG TAT TAC AGC GTC TTT GAC AGG GCC AAC AAC
1083

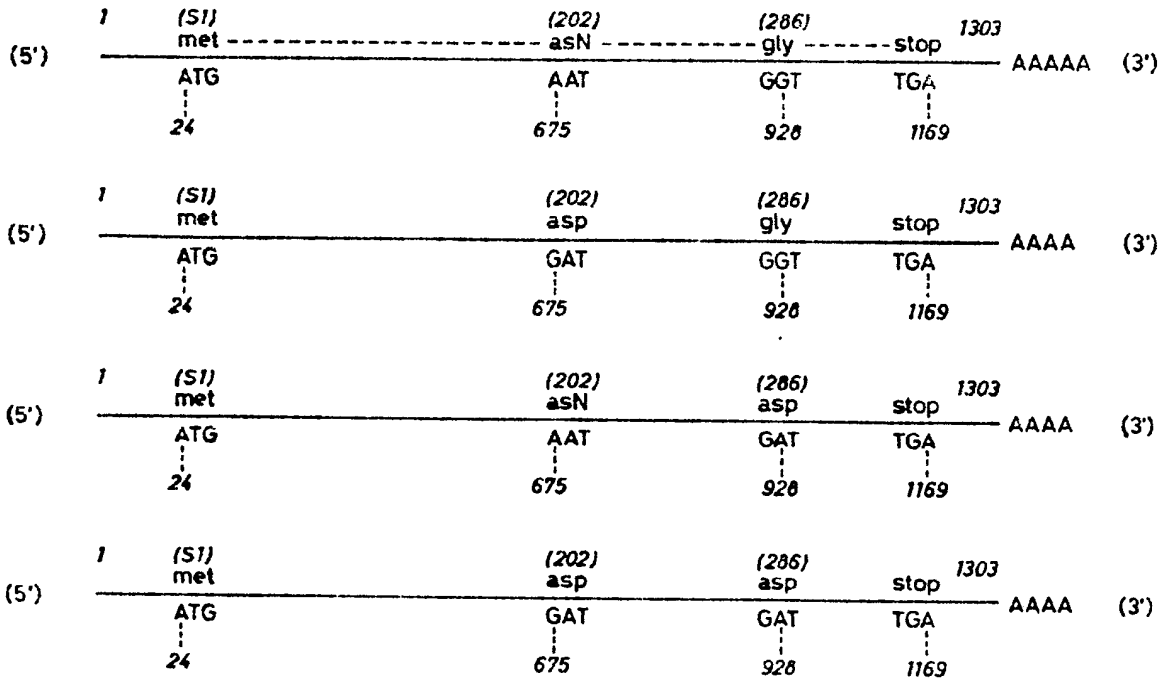
        360                 365
LEU VAL GLY LEU ALA LYS ALA ILE ***
CTC GTG GGG CTG GCC AAA GCC ATC TGA TCA CAT CGC TGA CCA AGA ACC TCA CTG TCC CCA
1143

CAC ACC TGC ACA CAC ACA TGC ACA CAT GTA CAT GAG CAC ATG TGC ACA CAC ACA GAT GAG
1203

GTT TCC AGA CAG ATG ATT CTC AAT AAA CGT TGT CTT TCT GCA AAA AAA A
1263                                                    1303
```

Fig. 1, continued.

Fig.2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met
(202)
asN
(286)
gly
stop
1303
(5')
AAAAA
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ATG
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GGT
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24
675
926
1169
1
(S1)
met
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asp
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gly
stop
1303
(5')
AAAA
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24
675
926
1169

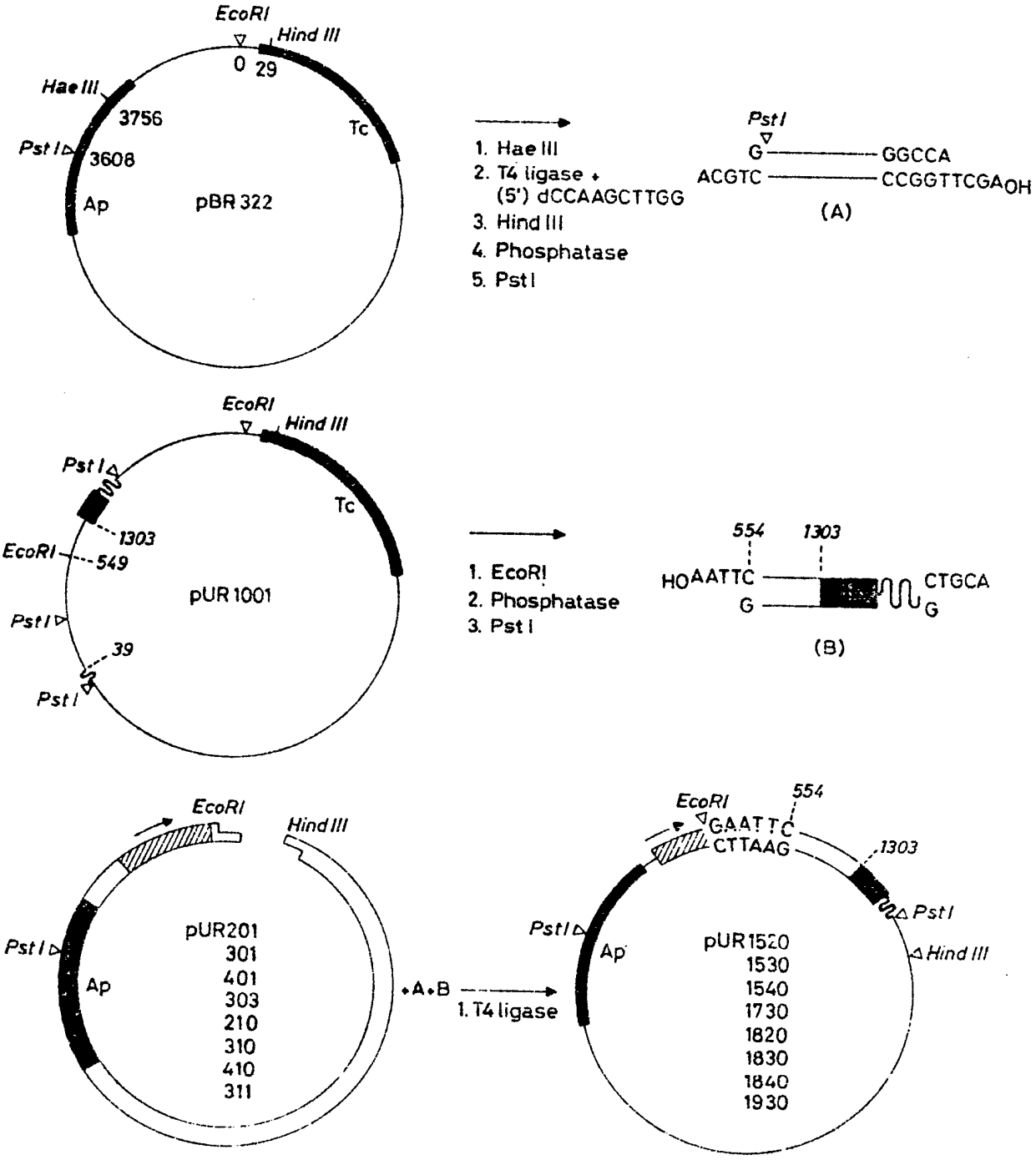
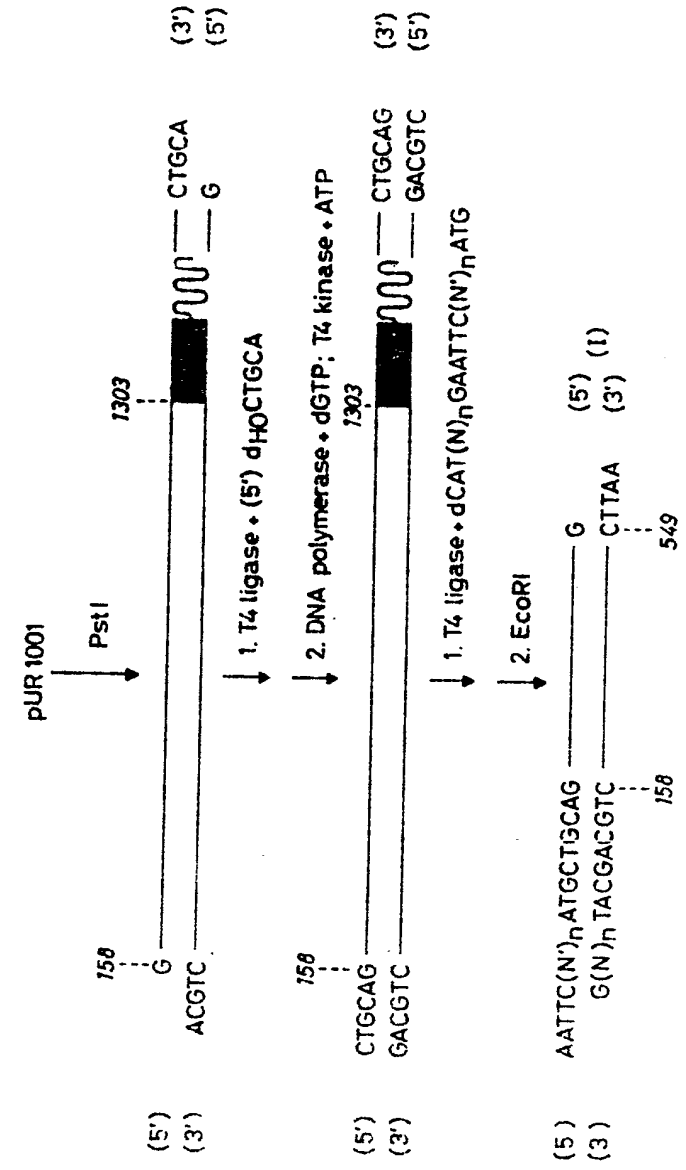

EcoRI
Hind III
0
29
Hae III
3756
Tc
Pst I
3608
Ap
pBR 322
1. Hae III
2. T4 ligase +
(5') dCCAAGCTTGG
3. Hind III
4. Phosphatase
5. Pst I
Pst I
G
GGCCA
ACGTC
CCGGTTCGA OH
(A)
EcoRI
Hind III
Pst I
100
1303
EcoRI
549
Tc
pUR 1001
Pst I
39
Pst I
1. EcoRI
2. Phosphatase
3. Pst I
554
1303
HO AATTC
CTGCA
G
G
(B)
EcoRI
Hind III
Pst I
Ap
pUR201
301
401
303
210
310
410
311
+A+B
1. T4 ligase
EcoRI
554
GAATTC
CTTAAG
1303
Pst I
Pst I
Ap
Hind III
pUR1520
1530
1540
1730
1820
1830
1840
1930

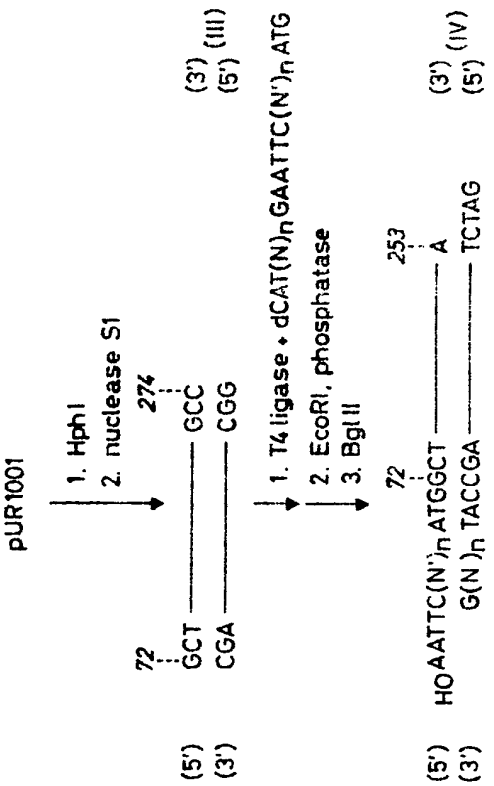
Fig. 4

0077109



pUR1001
Pst I
isolate 1300 bp fragment
1. heat denature
2. anneal with dGGGGAGGTGG
198
1303
GGGGAGGTGG
(3')
(5')
ACGTC
G
1. DNA polymerase
2. nuclease S1
198
1303
GGGGAGGTGG
(3')
(5')
CCCCTCCACC
C
G
1. T4 ligase + dCAT(N)nGAATTC(N')nATG
2. EcoRI, phosphatase
3 BglII
198
253
HO AATTC(N')n ATGGGGGAGGTGG
A
(3')
(5')
(II)
G(N)n TACCCCCTCCACC
TCTAG

Fig.6


pUR1001
1. HphI
2. nuclease S1
274
72
GCT
CGA
GCC
CGG
(5')
(3')
(3')
(5')
(III)
1. T4 ligase + dCAT(N)nGAATTC(N')nATG
2. EcoRI, phosphatase
3. BglII
72
253
HO AATTC(N')n ATGGCT
G(N)n TACCGA
A
TCTAG
(5')
(3')
(3')
(5')
(IV)

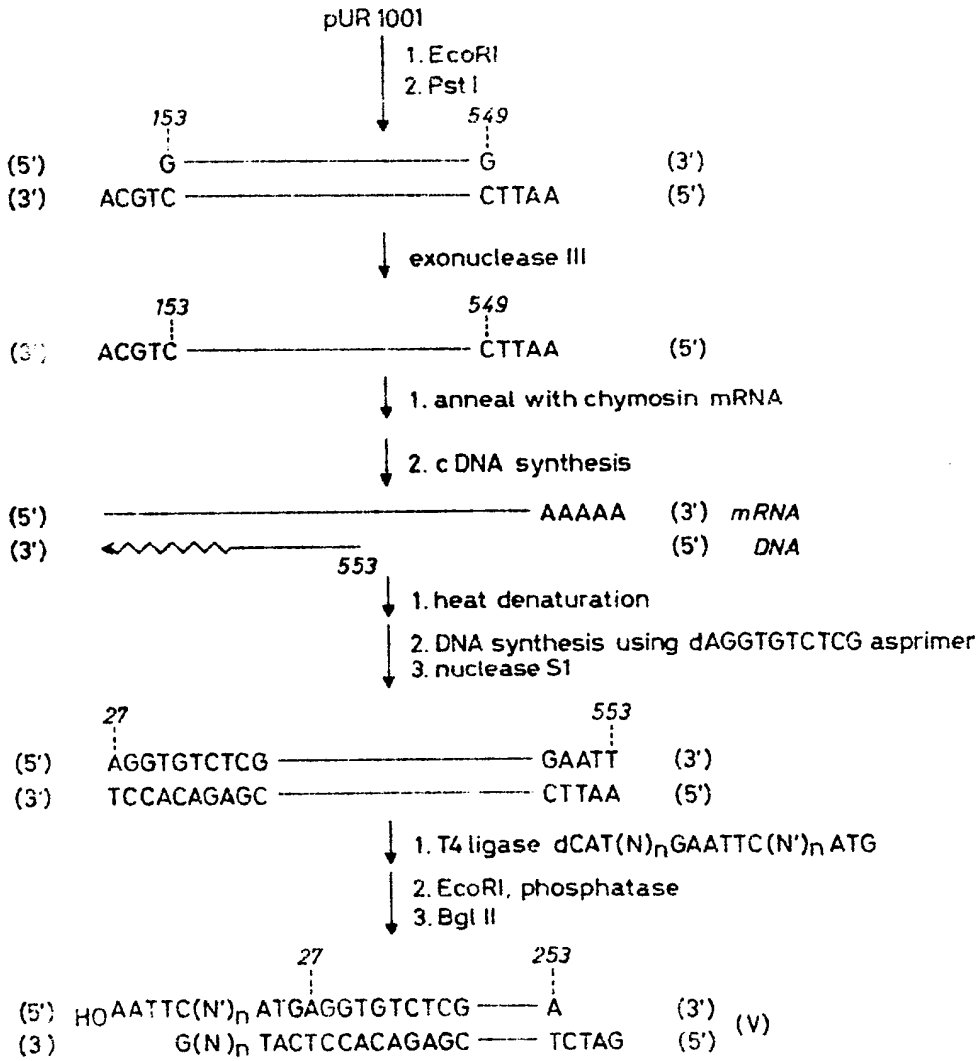

pUR 1001
1. EcoRI
2. Pst I
153
549
(5') G G (3')
(3') ACGTC CTTAA (5')
exonuclease III
153
549
(3') ACGTC CTTAA (5')
1. anneal with chymosin mRNA
2. c DNA synthesis
(5') AAAAA (3') mRNA
(3') (5') DNA
553
1. heat denaturation
2. DNA synthesis using dAGGTGTCTCG asprimer
3. nuclease S1
27
553
(5') AGGTGTCTCG GAATT (3')
(3') TCCACAGAGC CTTAA (5')
1. T4 ligase dCAT(N)nGAATTC(N')n ATG
2. EcoRI, phosphatase
3. Bgl II
27
253
(5') HO AATTC(N')n ATGAGGTGTCTCG A (3')
(3') G(N)n TACTCCACAGAGC TCTAG (5')
(V)

Fig. 8

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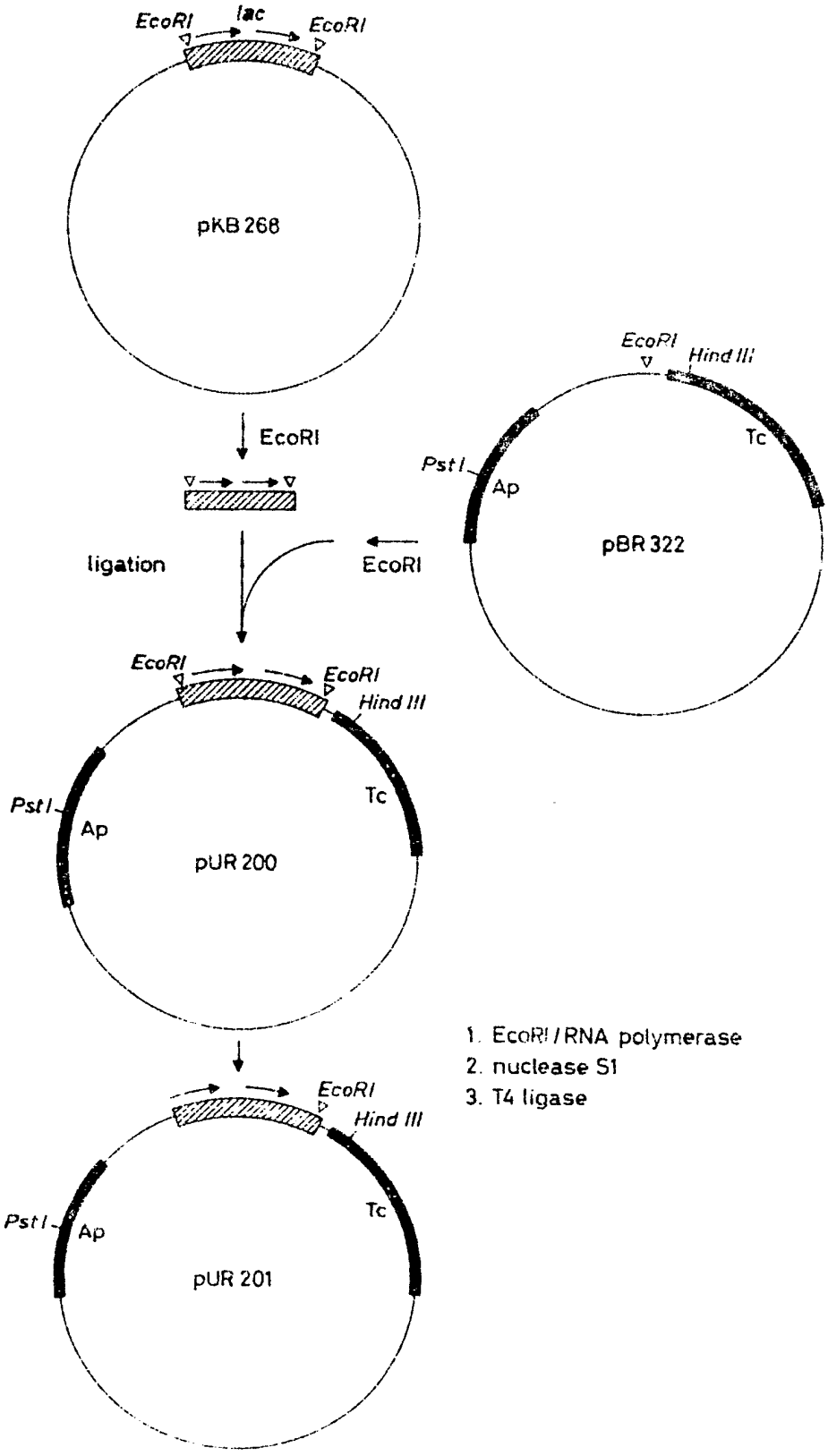
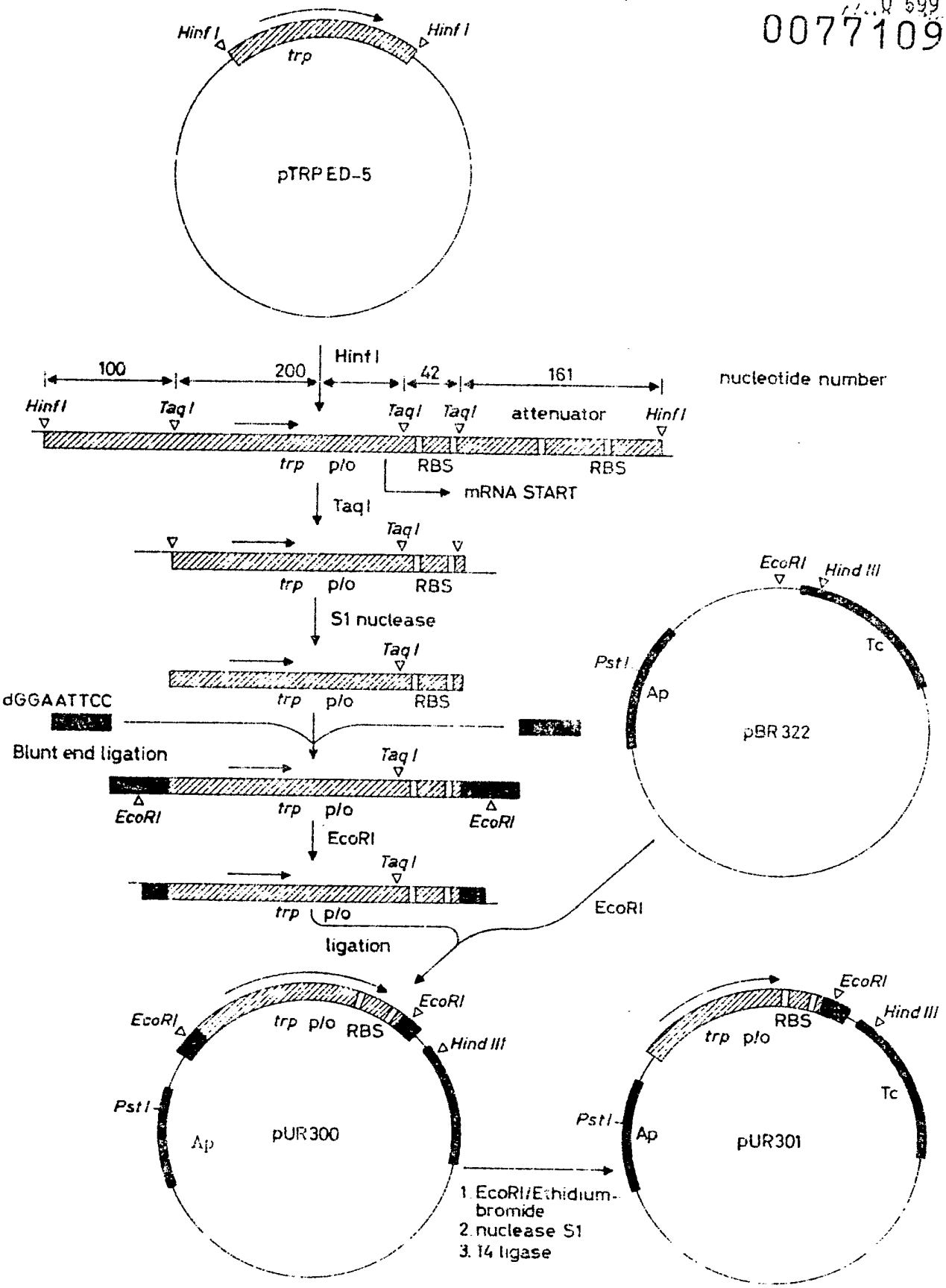
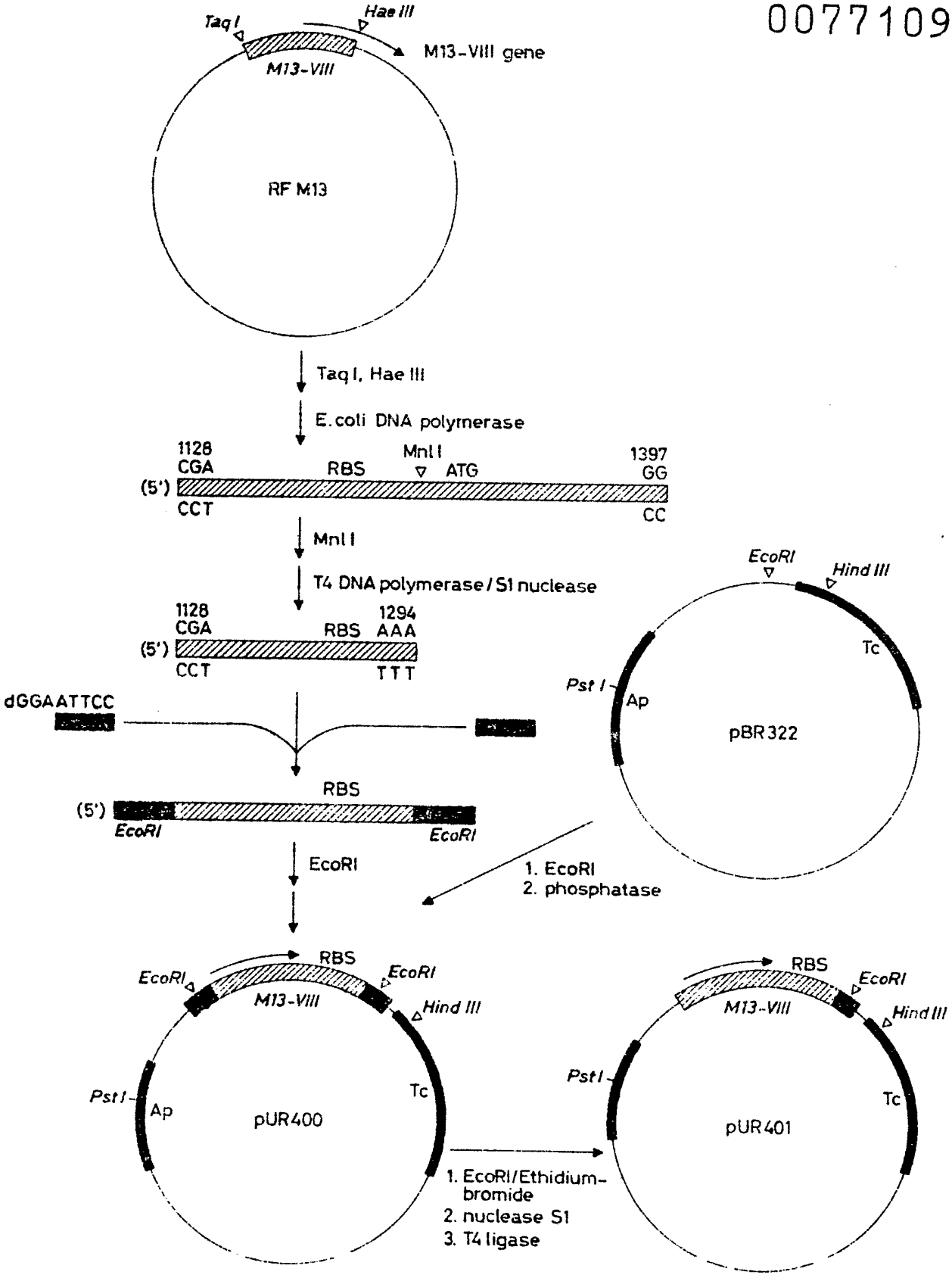


1. EcoRI / RNA polymerase
2. nuclease S1
3. T4 ligase


Hinf I
Hinf I
trp
pTRP ED-5
100
200
Hinf I
42
161
nucleotide number
Hinf I
Taq I
Taq I
Taq I
attenuator
Hinf I
trp
p/o
RBS
RBS
mRNA START
Taq I
Taq I
trp
p/o
RBS
S1 nuclease
Taq I
trp
p/o
RBS
dGGAATTCC
Blunt end ligation
Taq I
EcoRI
trp
p/o
EcoRI
EcoRI
Taq I
trp
p/o
ligation
EcoRI
EcoRI
Hind III
Tc
Pst I
Ap
pBR 322
EcoRI
trp p/o
RBS
Hind III
Pst I
Ap
pUR300
1. EcoRI/Ethidium-bromide
2. nuclease S1
3. T4 ligase
EcoRI
RBS
Hind III
trp p/o
Tc
Pst I
Ap
pUR301


Taq I
Hae III
M13-VIII gene
M13-VIII
RF M13
Taq I, Hae III
E.coli DNA polymerase
1128
CGA
RBS
MnI I
ATG
1397
GG
(5')
CCT
CC
MnI I
T4 DNA polymerase / S1 nuclease
1128
CGA
RBS
1294
AAA
(5')
CCT
TTT
dGGAATTCC
RBS
(5')
EcoRI
EcoRI
EcoRI
EcoRI
Hind III
Tc
Pst I
Ap
pBR322
1. EcoRI
2. phosphatase
RBS
EcoRI
EcoRI
M13-VIII
Hind III
Pst I
Ap
Tc
pUR400
1. EcoRI/Ethidium-bromide
2. nuclease S1
3. T4 ligase
RBS
EcoRI
M13-VIII
Hind III
Pst I
Tc
pUR401

0077109

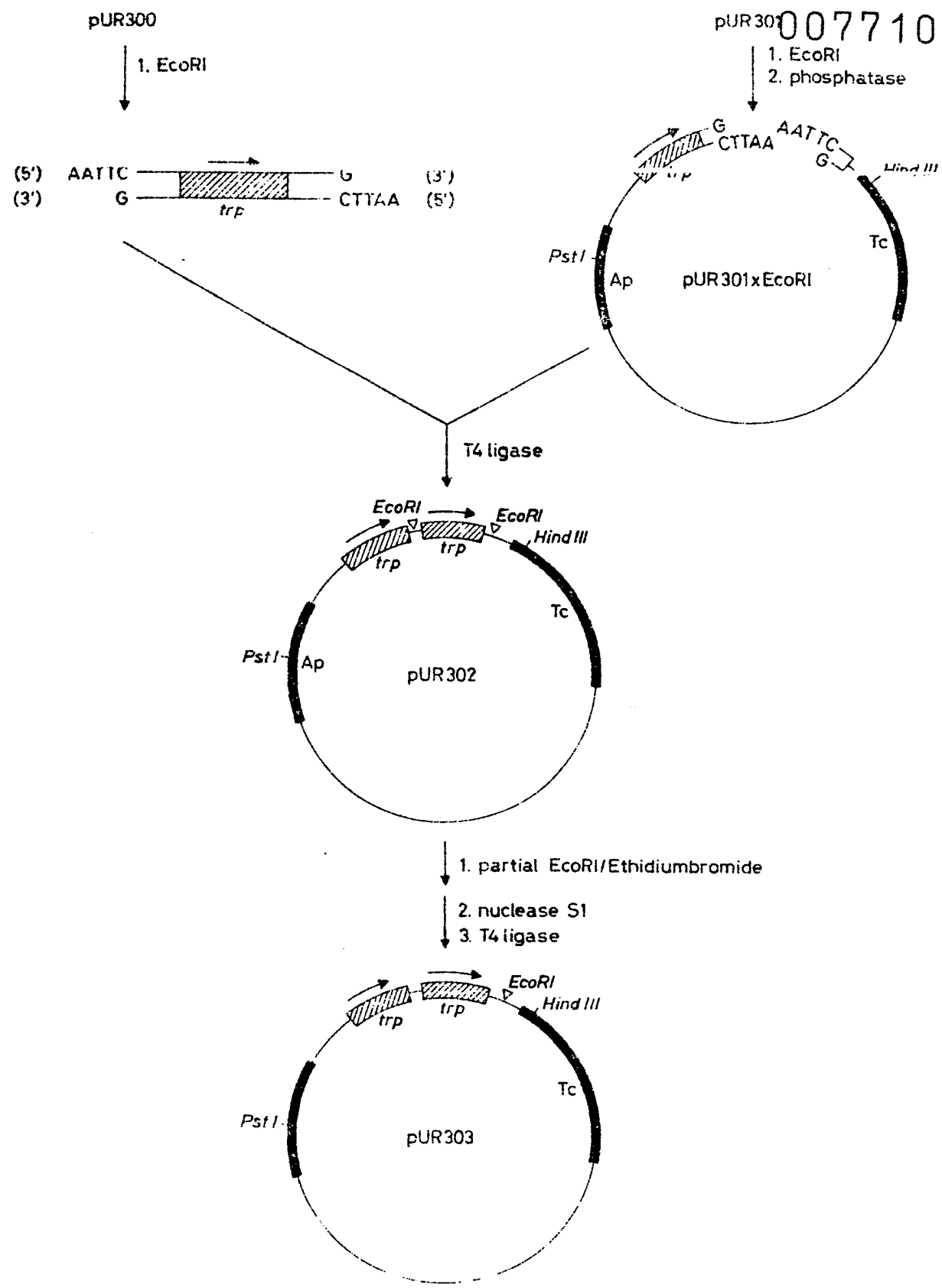

pUR300
1. EcoRI
(5') AATTC
(3') G
trp
G (3')
CTTAA (5')
pUR301
1. EcoRI
2. phosphatase
G
CTTAA
AATTC
G
Hind III
Tc
Pst I
Ap
pUR301xEcoRI
T4 ligase
EcoRI
EcoRI
Hind III
trp
trp
Tc
Pst I
Ap
pUR302
1. partial EcoRI/Ethidiumbromide
2. nuclease S1
3. T4 ligase
EcoRI
Hind III
trp
trp
Tc
Pst I
pUR303

Fig. 12

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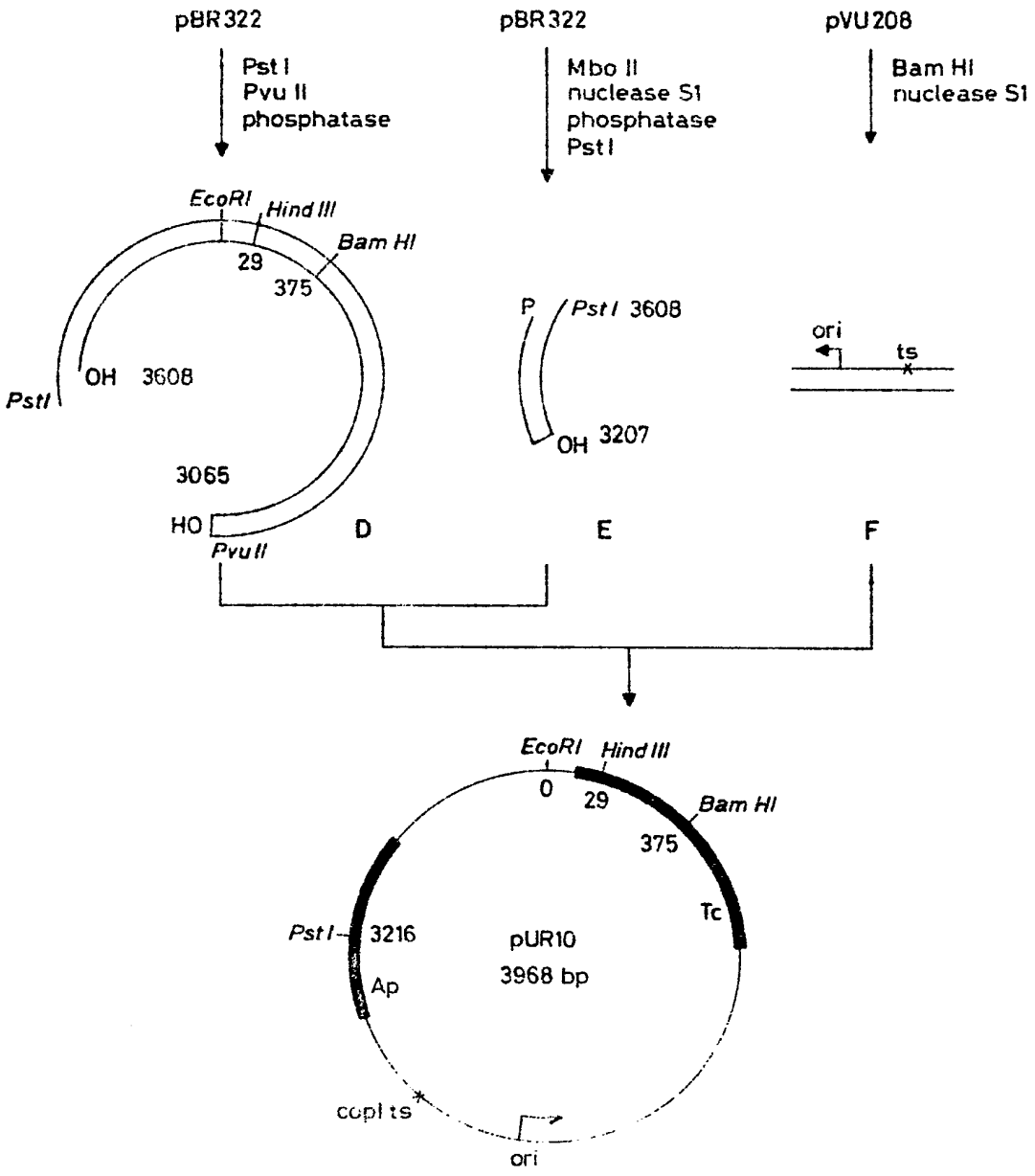
Q 599 (R)

0077109



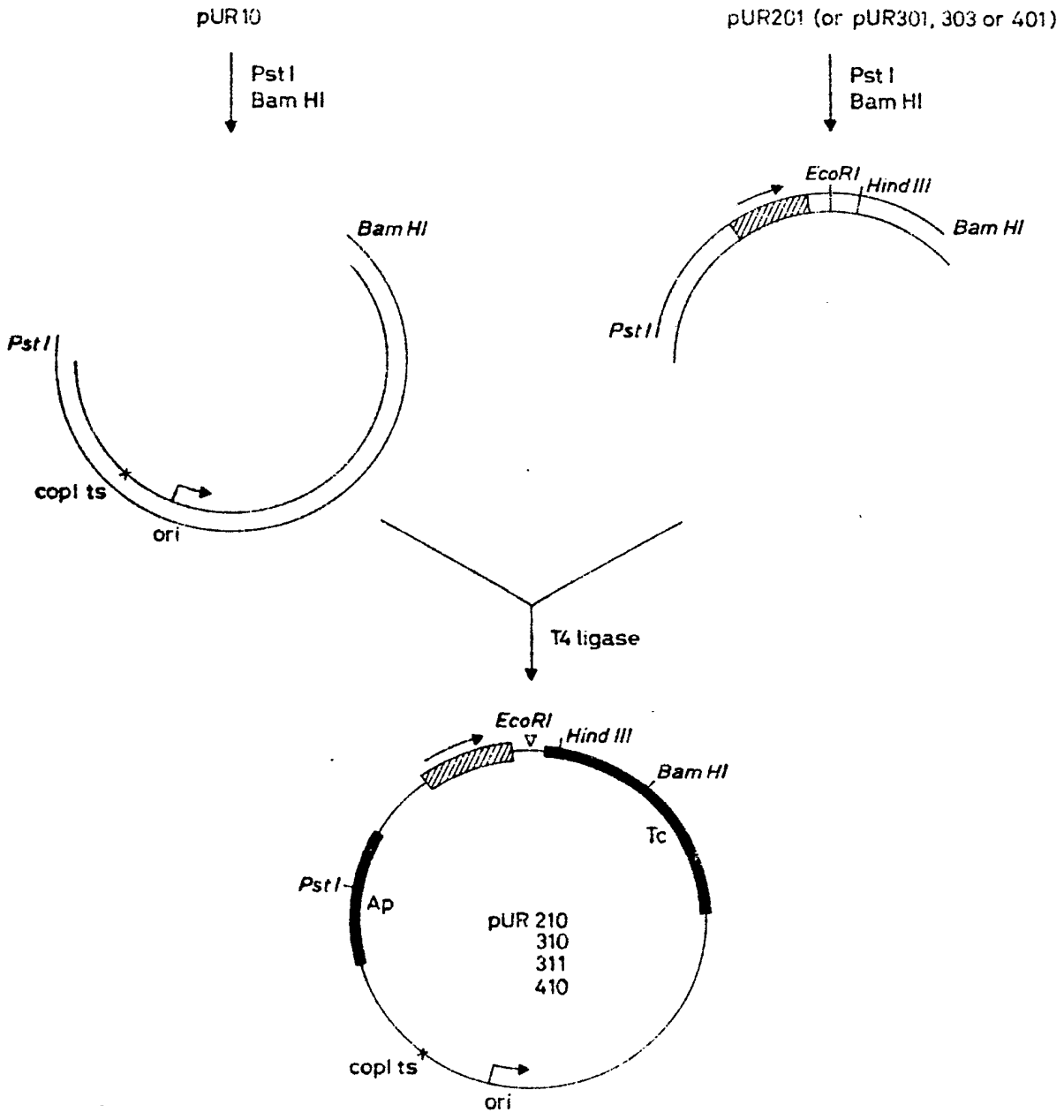
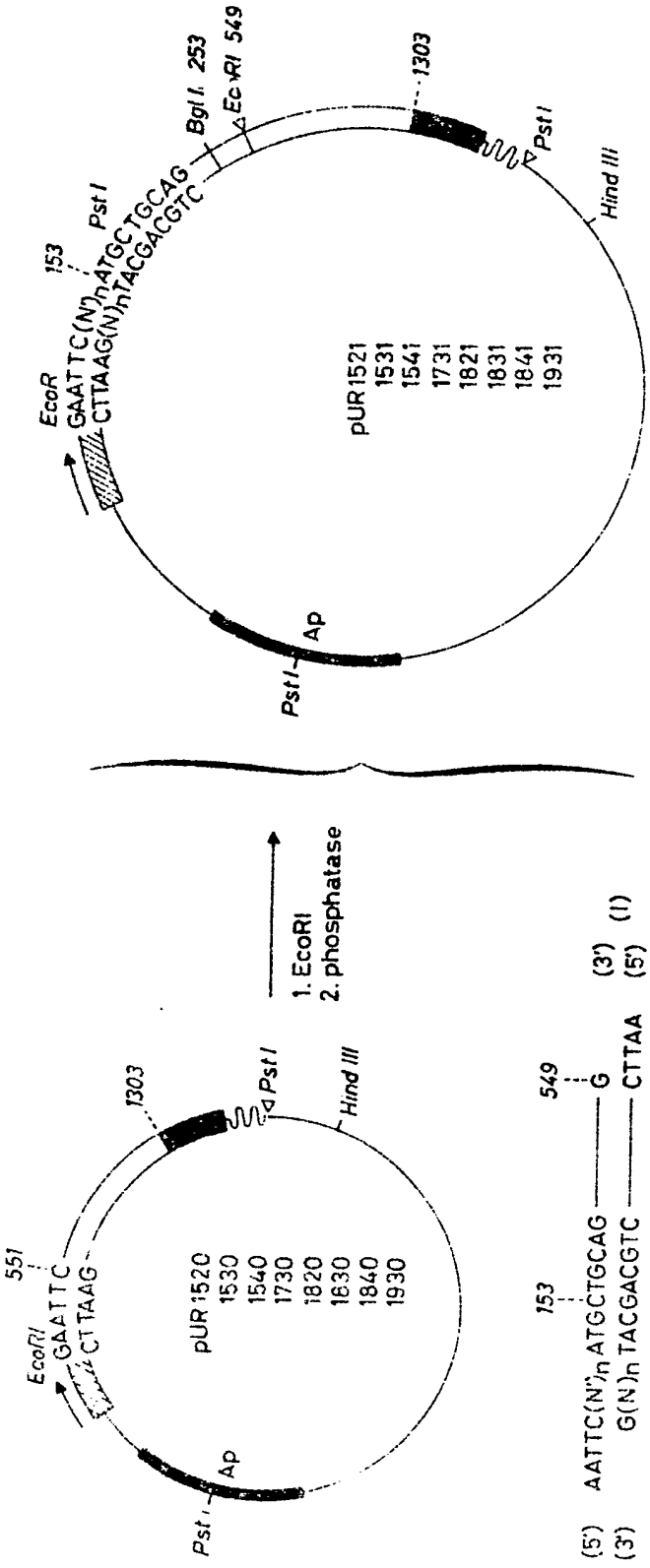
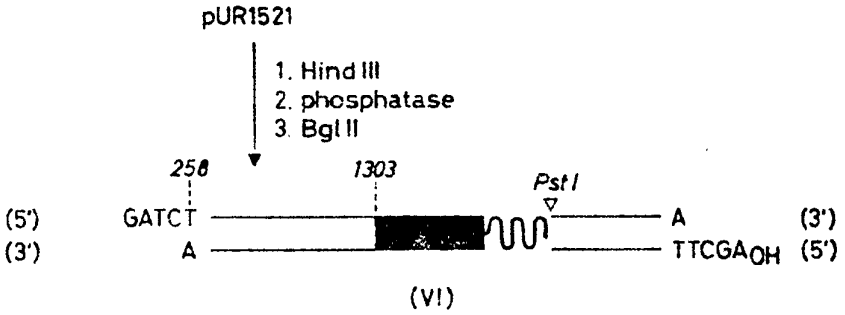

pUR10
Pst I
Bam HI
pUR201 (or pUR301, 303 or 401)
Pst I
Bam HI
EcoRI
Hind III
Bam HI
Pst I
Bam HI
Pst I
copI ts
ori
T4 ligase
EcoRI
Hind III
Bam HI
Tc
Pst I
Ap
pUR 210
310
311
410
copI ts
ori

Fig. 14

0077109
Q 599 (R)





pUR 201, 301, 401, 303, 210, 310, 410, 311

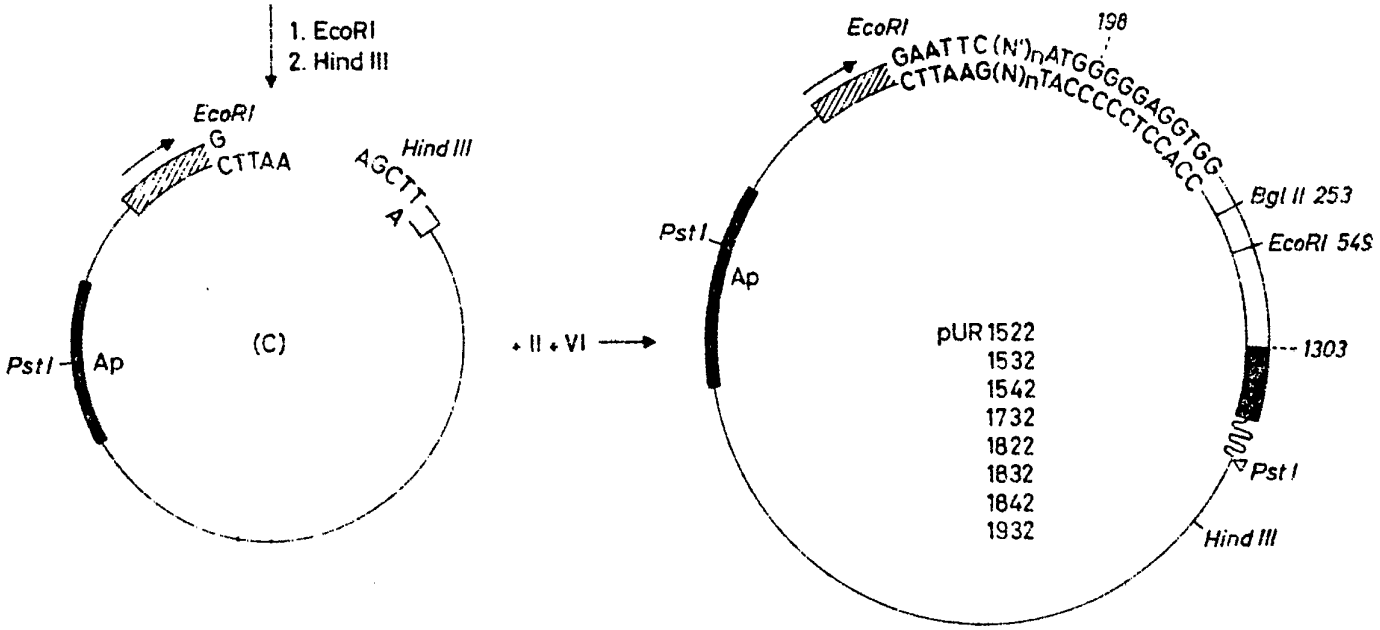


Fig. 16

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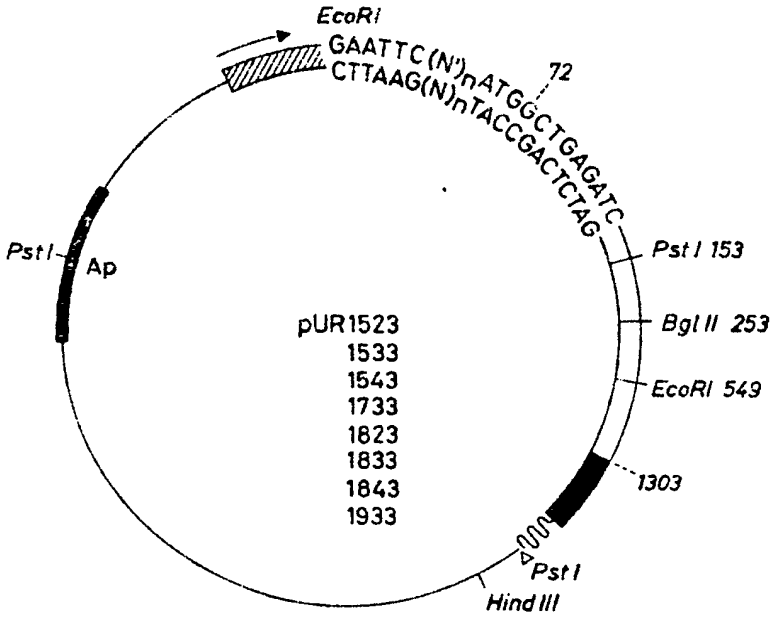
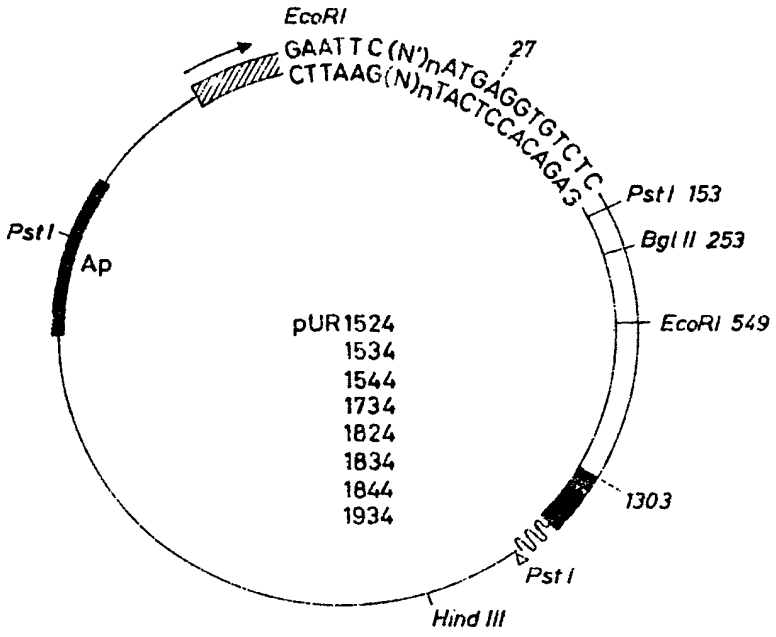
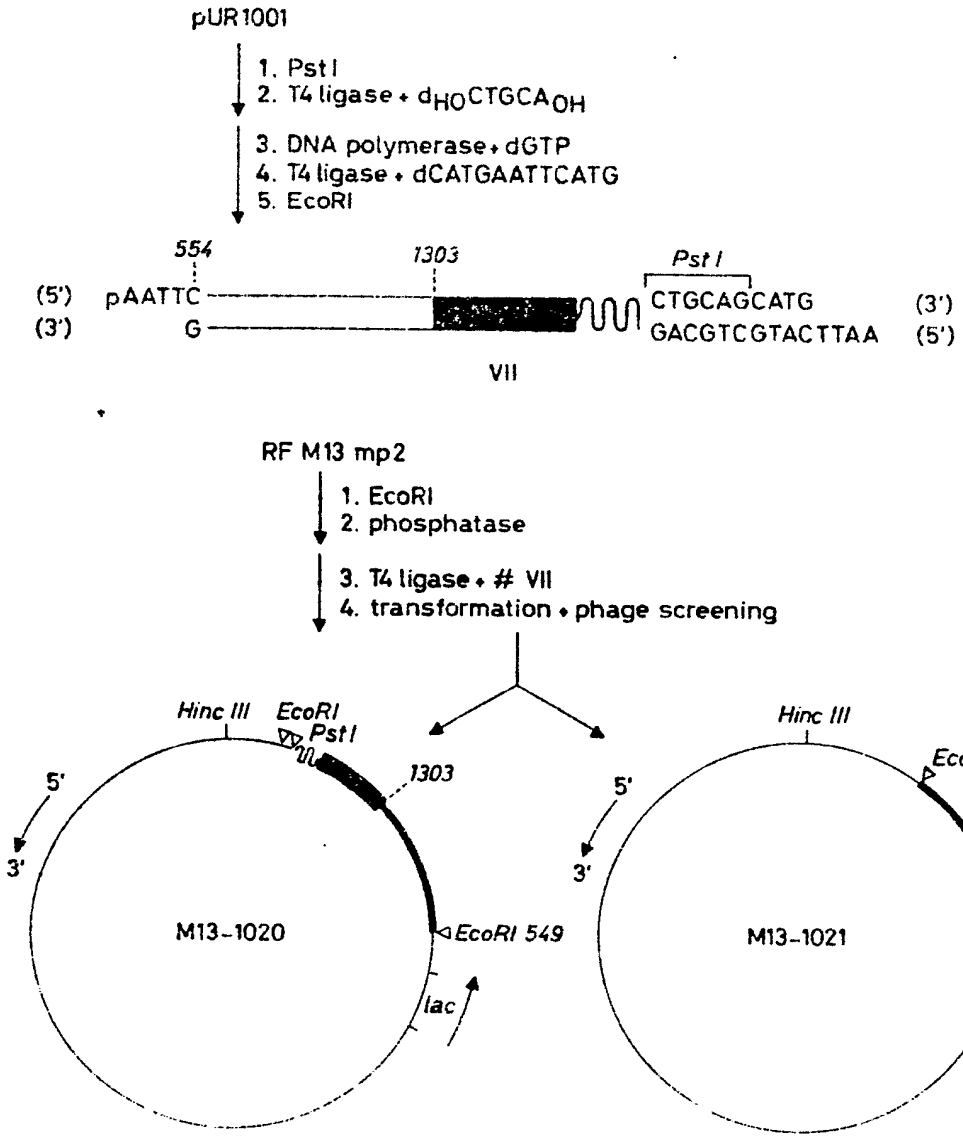
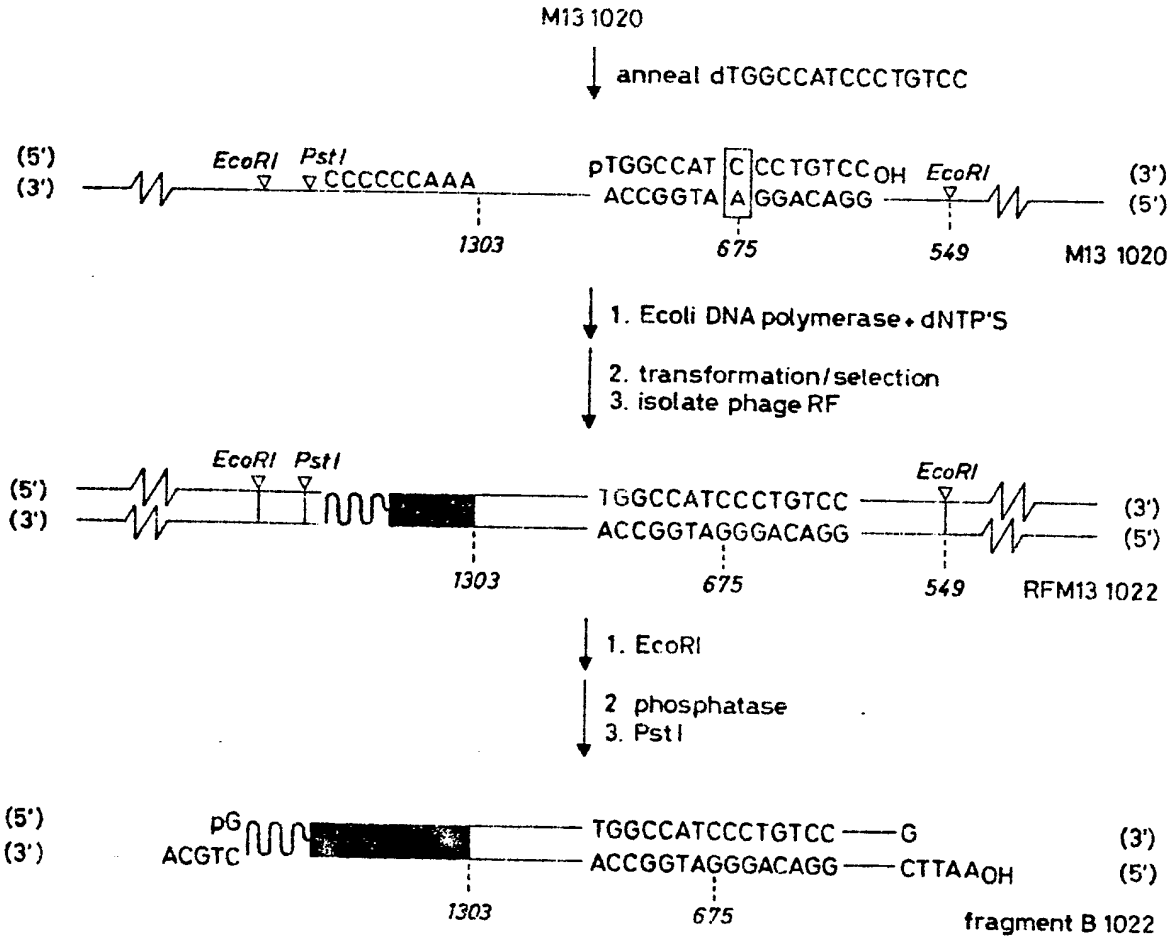


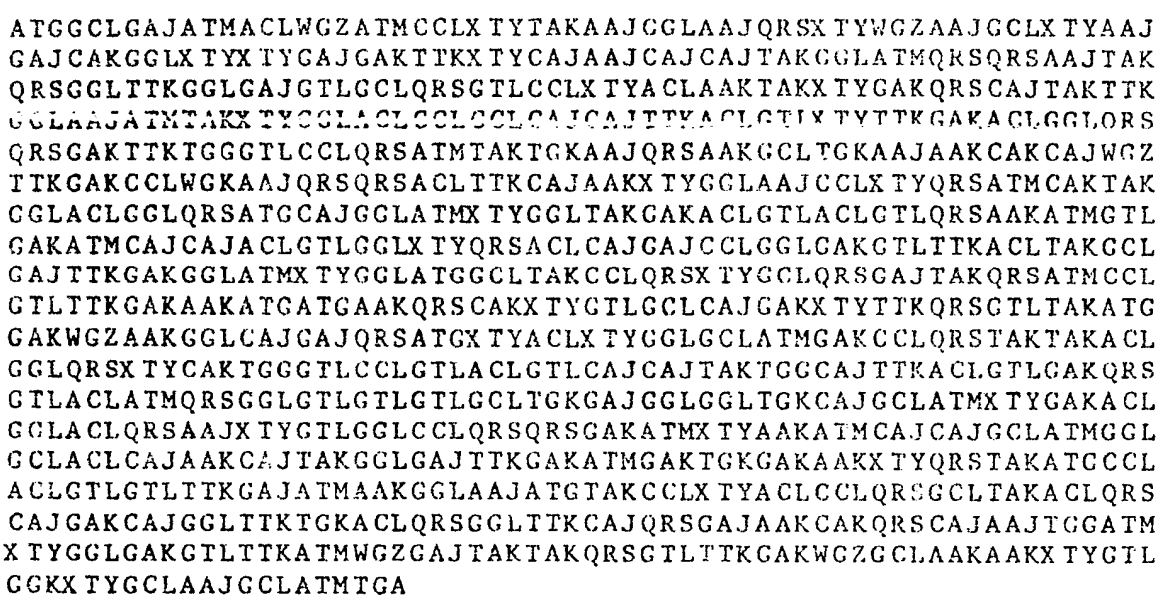

EcoRI
GAATTC(N')nATGAGGTGTCTC
CTTAAG(N)nTACTCCACAGAG
27
PstI 153
BglII 253
EcoRI 549
1303
PstI
HindIII
PstI
Ap
pUR1524
1534
1544
1734
1824
1834
1844
1934


pUR1001
1. PstI
2. T4 ligase + $d_{HO}CTGCA_{OH}$
3. DNA polymerase + dGTP
4. T4 ligase + dCATGAATTCATG
5. EcoRI
554
1303
Pst I
(5') pAATTC
(3') G
CTGCAGCATG (3')
GACGTCGTACTTAA (5')
VII
RF M13 mp2
1. EcoRI
2. phosphatase
3. T4 ligase + # VII
4. transformation + phage screening
Hinc III
EcoRI
Pst I
1303
5'
3'
M13-1020
EcoRI 549
lac
Hinc III
EcoRI 549
1303
5'
3'
M13-1021
Pst I
EcoRI
lac


M13 1020
anneal dTGGCCATCCCTGTCC
(5')
(3')
EcoRI
PstI
CCCCCCAAA
pTGGCCAT C CCTGTCC OH
ACCGGTA A GGACAGG
EcoRI
(3')
(5')
1303
675
549
M13 1020
1. Ecoli DNA polymerase + dNTP'S
2. transformation/selection
3. isolate phage RF
EcoRI
PstI
TGGCCATCCCTGTCC
ACCGGTAGGGACAGG
EcoRI
(5')
(3')
(3')
(5')
1303
675
549
RFM13 1022
1. EcoRI
2 phosphatase
3. PstI
(5')
(3')
pG
ACGTC
TGGCCATCCCTGTCC — G
ACCGGTAGGGACAGG — CTTAA OH
(3')
(5')
1303
675
fragment B 1022

ATGWGZTGKXTYGTLGTLXTYXTYGCLGTLTTKGCLXTYQRSCAJGGLGCLGAJATMACL
WGZATMCCLXTYTAKAAJGGLAAJQRSXTYWGZAAJGCLXTYAAJGAJCAKGGLXTYXTY
GAJGAKTTKXTYCAJAAJCAJCAJTAKGGLATMQRSQRSAAJTAKQRSGGLTTKGGLGAJ
GTLGCLQRSGTLCCLXTYACLAAKTAKXTYGAKQRSCAJTAKTTKGGLAAJATMTAKXTY
GGLACLCCLCCLCAJGAJTTKACLGTLXTYTTKGAKACLGCLQRSQRSGAKTTKTGGGTL
CCLQRSATMTAKTGKAAJQRSAAKGCLTGKAAJAAKCAKCAJWGZTTKGAKCCLWGKAAJ
QRSQRSACLTTKCAJAAKXTYGGLAAJCCLXTYQRSATMCAKTAKGGLACLGGLQRSATG
CAJGGLATMXTYGGLTAKGAKACLGTLACLGTLQRSAAKATMGTLGAKATMCAJCAJACL
GTLGGLXTYQRSACLCAJGAJCCLGGLGAKGTLTTKACLTAKGCLGAJTTKGAKGGLATM
XTYGGLATGGCLTAKCCLQRSXTYGGLQRSGAJTAKQRSATMCCLGTLTTKGAKAAKATG
ATGAAKQRSCAKXTYGTLGCLCAJGAKXTYTTKQRSGTLTAKATGGAKWGZAAKGGLCAJ
GAJQRSATGXTYACLXTYGGLGCLATMGAKCCLQRSTAKTAKACLGGLQRSXTYCAKTGG
GTLCCLGTLACLGTLCAJCAJTAKTGGCAJTTKACLGTLGAKQRSGTLACLATMQRSGGL
GTLGTLGTLGCLTGKGAJGGLGGLTGKCAJGCLATMXTYGAKACLGGLACLQRSAAJXTY
GTLGGLCCLQRSQRSGAKATMXTYAAKATMCAJCAJGCLATMGGLGCLACLCAJAAKCAJ
TAKGGLGAJTTKGAKATMGAKTGKGAKAAKXTYQRSTAKATGCCLACLGTLGTLTTKGAJ
ATMAAKGGLAAJATGTAKCCLXTYACLCCLQRSGCLTAKACLQRSCAJGAKCAJGGLTTK
TGKACLQRSGGLTTKCAJQRSGAJAAKCAKQRSCAJAAJTGGATMXTYGGLGAKGTLTTK
ATMWGZGAJTAKTAKQRSGTLTTKGAKWGZGCLAAKAAKXTYGTLGGKXTYGCLAAJGCL
ATMTGA

Fig. 19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GAJCAKGGLX TYX TYGAJGAKTTKX TYCAJAAJCAJCAJTAKCGLATMQRSQRSAAJTAK
QRSGGLTTKGGLGAJGTLGCLQRSGTLCCLX TYACLAAKTAKX TYGAKQRSCAJTAKTTK
GGLAAJATMTAKX TYCGLACLCCLCCLCAJCAJTTKACLGTLX TYTTKGAKACLGGLQRS
QRSGAKTTKTGGGTLCCLQRSATMTAKTGKAAJQRSAAKGCLTGKAAJAAKCAKCAJWGZ
TTKGAKCCLWGKAAJQRSQRSACLTTKCAJAAKX TYGGLAAJCCLX TYQRSATMCAKTAK
CGLACLGGLQRSATGCAJGGLATMX TYGGLTAKGAKACLGTLACLGTLQRSAAKATMGTL
GAKATMCAJCAJACLGTLGGLX TYQRSACLCAJGAJCCLGGLGAKGTLTTKACLTAKGCL
GAJTTKGAKGGLATMX TYGGLATGGCLTAKCCLQRSX TYGCLQRSGAJTAKQRSATMCCL
GTLTTKGAKAAKATGATGAAKQRSCAKX TYGTLGCLCAJGAKX TYTTKQRSGTLTAKATG
GAKWGZAAKGGLCAJGAJQRSATGX TYACLX TYGGLGCLATMGAKCCLQRSTAKTAKACL
GGLQRSX TYCAKTGGGTLCCLGTLACLGTLCAJCAJTAKTGGCAJTTKACLGTLGAKQRS
GTLACLATMQRSGGLGTLGTLGTLGCLTGKGAJGGLGGLTGKCAJGCLATMX TYGAKACL
GGLACLQRSAAJX TYGTLGGLCCLQRSQRSGAKATMX TYAAKATMCAJCAJGCLATMGGL
GCLACLCAJAAKCAJTAKGGLGAJTTKGAKATMGAKTGKGAKAAKX TYQRSTAKATGCCL
ACLGTLGTLTTKGAJATMAAKGGLAAJATGTAKCCLX TYACLCCLQRSGCLTAKACLQRS
CAJGAKCAJGGLTTKTGKACLQRSGGLTTKCAJQRSGAJAAKCAKQRSCAJAAJTGGATM
X TYGGLGAKGTLTTKATMWGZGAJTAKTAKQRSGTLTTKGAKWGZGCLAAKAAKX TYGTL
GGKX TYGCLAAJGCLATMTGA

Fig. 20

ATGXTYCAJAAJCAJCAJTAKGGLATMQRSQRSAAJTAKQRSGGLTTKGGLGAJGTLGCL
QRSGTLCCLXTYACLAAKTAKXTYGAKQRSCAJTAKTTKGGLAAJATMTAKXTYGGLACL
CCLCCLCAJGAJTTKACLGTLXTYTTKGAKACLGGLQRSQRSGAKTTKTGGGTLCCLQRS
ATMTAKTGKAAJQRSAAKGCLTGKAAJAAKCAKCAJWGZTTKGAKCCLWGKAAJQRSQRS
ACLTTKCAJAAKXTYGGLAAJCCLXTYQRSATMCAKTAKGGLACLGGLQRSATGCAJGGL
ATMXTYGGLTAKGAKACLGTLACLGTLQRSAAKATMGTLGAKATMCAJCAJACLGTLGGL
XTYQRSACLCAJGAJCCLGGLGAKGTLTTKACLTAKGCLCAJTTKGAKGGLATMXTYGGL
ATGGCLTAKCCLQRSXTYGCLQRSGAJTAKQRSATMCCLGTLTTKGAKAAKATGATGAAK
QRSCAKXTYGTLGCLCAJGAKXTYTTKQRSGTLTAKATGGAKWGZAAKGGLGAJGA.QRS
ATGXTYACLXTYGGLGCLATMGAKCCLQRSTAKTAKACLGGLQRSXTYCAKTGGGTLCCL
GTLACLGTLCAJCAJTAKTGGCAJTTKACLGTLGAKQRSGTLACLATMQRSGGLGTLGTL
GTLGCLTGKGAJGGLGGLTGKCAJGGLATMXTYGAKACLGGLACLQRSAAJXTYGTLGGL
CCLQRSQRSGAKATMXTYAAKATMCAJCAJGGLATMGGLGGLACLGAJAAKCAJTAKGGL
GAJTTKGAKATMGAKTGKGAKAAKXTYQRSTAKATGCCLACLGTLGTLTTKGAJATMAAK
GGLAAJATGTAKCCLXTYACLCCLQRSGGLTAKACLQRSCAJGAKCAJGGLTTKTGKACL
QRSGGLTTKCAJQRSGAJAAKCAKQRSCAJAAJTGGATMXTYGGLGAKGTLTTKATMWGZ
GAJTAKTAKQRSGTLTTKGAKWGZGCLAAKAAKXTYGTLGGKXTYGCLAAJGCLATMTGA

Fig. 21

ATGGGLCAJGTLGCLQRSGTLCCLX TYACLAAKTAKX TYGAKQRSCAJTAKTTKGGLAAJ
ATMTAKX TYGGLACLCCLCCLCAJCAJTTKACLGTLX TYTTKGAKACLGGLQRSQRSGAK
TTKTGGGTLCCLQRSATMTAKTGKAAJQRSAAKGGLTGKAAJAAKCAKCAJWGZTTKGAK
CCLWGKAAJQRSQRSACLTTKCAJAAKX TYGGLAAJCCLX TYQRSATMCAKTAKGGLACL
CGLQRSATGCAJGGLATMX TYGGLTAKCAKACLGTLACLGTLQRSAAKATMGTLGAKATM
CAJCAJACLGTLGGLX TYQRSACLCAJGAJCCLGGLCAKGTLTTKACLTAKGCLCAJTTK
GAKGGLATMX TYGGLATGGCLTAKCCLQRSX TYGCLQRSGAJTAKQRSATMCCLGTLTTK
GAKAAKATGATGAAKQRSCAKX TYGTLGCLCAJGAKX TYTTKQRSGTLTAKATGGAKWGZ
AAKGGLCAJGAJQRSATGX TYACLX TYGGLGCLATMGAKCCLQRSTAKTAKACLGGLQRS
X TYCAKTGGGTLCCLGTLACLGTLCAJCAJTAKTGGCAJTTKACLGTLGAKQRSGTLACL
ATMQRSGGLGTLGTLGTLGCLTGKGAJGGLGGLTGKCAJGCLATMX TYGAKACLGGLACL
QRSAAJX TYGTLGGLCCLQRSQRSGAKATMX TYAAKATMCAJCAJGCLATMCGLGCLACL
CAJAAKCAJTAKGGLGAJTTKGAKATMGAKTGKGAKAAKX TYQRSTAKATGCCLACLGTL
GTLTTKGAJATMAAKGGLAAJATGTAKCCLX TYACLCCLQRSGCLTAKACLQRSCAJGAK
CAJGGLTTKTGKACLQRSGGLTTKCAJQRSGAJAAKCAKQRSCAJAAJTGGATMX TYGGL
GAKGTLTTKATMWGZGAJTAKTAKQRSGTLTTKGAKWGZGCLAAKAAKX TYGTLGGKX TY
GCLAAJGCLATMTGA

Fig. 22